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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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TITLE OF THE INVENTION (280 characters max)

Regulated Attenuation of Live Vaccines to Enhance Cross-protective Immunogenicity

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒	Specification *Number of Pages*	45	☒	Seq. List w/Disc	11 Pgs
☒	Drawing(s) *Number of Sheets*	11	☒	Other (specify)	Claims: 4 Pages

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: 2001-02944 (US Dept. of Agriculture) and DE06669 (National Institute of Health)

Respectfully submitted,

SIGNATURE Daniel A Kasten Date 4/15/02

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TELEPHONE 314-552-6305 Docket Number: 56029/31335

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PATENT

Regulated Attenuation Of Live Vaccines To Enhance Cross-protective Immunogenicity

REFERENCE TO GOVERNMENT GRANT

This invention was made with government support under Grant No. 2001-02944 by the United States Department of Agriculture and/or Grant No. DE06669 by the National Institutes of Health. The United States government may have certain rights in the invention.

SEQUENCE LISTING

This application contains a paper copy of a Sequence Listing and appended hereto is a computer readable form of the same Sequence Listing, which is hereby incorporated by reference. The sequence listing information recorded in computer readable form is identical to the written sequence listing.

FIELD OF THE INVENTION

The invention relates generally to the field of recombinant attenuated bacteria, and more specifically to construction of bacterial strains which have the ability to induce immune responses that result in protection against infection by a diversity of bacterial serotypes and species.

BACKGROUND OF THE INVENTION

Citations to some documents may be indicated as numbers in parentheses; those numbers refer to the bibliography under the heading "Related Art" at the end of this section. Those references, as well as others cited in this document are hereby incorporated by reference.

Live bacterial vaccine vectors have been used successfully to elicit effective immune responses in order to prevent infection. Such vectors have been used to induce protective immunity against infection from homologous and heterologous bacterial strains. Live attenuated bacterial vectors are also useful for food safety, for example to prevent or reduce infection of livestock animals such as poultry or cattle by bacterial strains that are pathogenic to humans, such as *Salmonella* or *E. coli*.

The ability of live attenuated pathogenic bacteria of the Enterobacteriaceae family to colonize the gut-associated lymphoid tissue (GALT; Peyer's patches) and the deep tissues following oral administration is beneficial in that it stimulates all arms of the immune response, including mucosal, humoral and cellular immunities (Curtiss/Doggett/Nayak/Srinivasan 1996; Galan and Sansonetti 1996; Medina/Guzman 2001). Colonization of the intestinal tract by gram negative bacteria is dependent in part upon the expression of a number of surface antigens, including LPS O-antigen side chains, a diversity of fimbrial adhesins, flagella and certain outer membrane proteins. Thus, rough mutants, i.e., those with little or no O-antigen on their LPS, that have mutational lesions precluding synthesis of LPS O-antigen or parts of the LPS core tend not to colonize the intestinal tract (Roantree, 1971; Nnalue, 1990) and are defective in attaching to and invading intestinal cells and surviving in cells on the other side of the intestinal wall barrier. (25, 26). This latter phenotype is due to the fact that LPS is needed for bacteria to display resistance to killing by macrophages (27, 28) and also for the display of serum resistance (29, 30), that is, the ability to multiply in blood. In accord with these observations, rough mutants defective in LPS synthesis and thus defective in infection are among the most frequently isolated using signature tagged mutagenesis (31) and genes for LPS biosynthesis are very often up-regulated during infection as revealed by use of in vivo expression technology (32). Rough mutants have generally not been very effective when used as live vaccines. (33, 34, Hill

abstract). Thus, it follows that an attenuated immunogenic live bacterial vaccine, to be safe and efficacious must not only display avirulence and not induce disease symptomology, but also must be able to reach, multiply and persist for a while in those lymphoid organs necessary to stimulate a protective immune response. Permanently rough strains cannot achieve the latter. The use of bacterial strains with mutations in the *galE* locus encoding UDP-galactose epimerase, an enzyme that interconverts UDP-glucose and UDP-galactose (UDP-gal) (35), has been considered as a way of overcoming the above limitation. UDP-gal is needed for the synthesis of both the LPS core and O-antigen in many bacterial strains. (36). When *Salmonella galE* mutants are provided low levels of galactose, they make normal LPS, but when deprived of galactose, they rapidly lose the ability to synthesize a complete LPS O-antigen and core. (37). One of the difficulties with *galE* mutants is that they are exceedingly sensitive to galactose (38, 39) and accumulate Gal-resistant mutants that are permanently rough and therefore not only avirulent, but also non immunogenic. Because of the LPS core defect, these *galE* mutants are somewhat hyper attenuated and do not induce high-level protective immunity. (40, 41). Another alternative to generate a reversibly rough phenotype is to make use of *pmi* mutants that have a mutation in the gene for phosphomannose isomerase (42), which interconverts mannose 6-phosphate and fructose 6-phosphate. Mannose 6-phosphate is then converted to GDP-mannose which is used for synthesis of O-antigen side chains (43). *pmi* mutants are not mannose sensitive and, as shown by Collins et al. (44), are attenuated and somewhat immunogenic. *pmi* mutants, when grown in media containing mannose, synthesize wild-type levels of LPS O-antigen side chains. In addition, *pmi* mutants do not lose the ability to synthesize LPS core.

Immune responses to iron-regulated outer membrane proteins (IROMPS) are known to be effective in preventing septicemic infection with enteropathogens. (Bolin 1987). Further, many bacterial serotypes and species in the Enterobacteriaceae family synthesize IROMPs and other proteins involved in iron uptake that share significant antigenic homology such that antibodies induced to proteins from one bacterial serotype or species are effective in binding to IROMPS and other iron uptake proteins from other serotypes and species. (Jun Lin 2001).

The *fur* gene encodes a repressor that represses all genes encoding IROMPS, in the presence of free iron. (Earhart 1996). When iron concentrations become low, as is the case in most animal host tissues beyond the intestinal wall barrier, the *fur* repression decreases and higher level expression of IROMPS and other *fur*-regulated genes needed to sequester iron is observed. *fur* mutants are attenuated when fed orally, giving a two to three log higher LD50 when administered either to mice (52) or day-of-hatch chicks. On the other hand, administering a *fur* mutant of S. typhimurium by the intraperitoneal route leads to only a slightly elevated LD50 compared to that of the wild-type parent. (53). In the intestinal tract iron is plentiful, both due to non absorption of dietary iron and the presence of iron from hemoglobin breakdown contributed into the intestinal tract as a component of bile. Green et al. 1968. It is also well known that iron, unless in a complex form, can promote the formation of damaging hydroxyl radicals, which may account, in part, for the toxicity of iron (51). Thus the high oral LD50 of *fur* mutants may be due to toxicity of free iron encountered in the intestinal tract. *fur* mutants are also acid sensitive (55) and are thus potentially sensitive to the gastric acidity barrier and to killing in acidified phagosomes in macrophages (56, 57). In summary, while *fur* mutant bacterial strains would display higher levels of IROMPs that likely would induce protective immunity, their avirulence properties when administered orally make them poor immunogens. So, while mutants unable to produce Fur are attenuated when delivered orally, because of substantial iron induced death they do not induce a significant immune response.

Members of the Enterobacteriaceae family cause a wide variety of human and animal diseases, including gram-negative sepsis, food poisoning, and typhoid fever. In addition, many farm animals are colonized with diverse enteric bacteria such as many serotypes of Salmonella without causing disease. Such bacteria are capable of transmission through the food chain to cause diseases in humans. Developing vaccines to prevent all the types of enteric diseases caused by bacterial enteric pathogens of diverse genera, species and serotypes and to prevent colonization by these diverse bacterial types in farm animals to enhance food safety would be prohibitively expensive. The incidence of these diseases and the prevalence of colonization of farm animals highlights the need for vaccines that would cross-protect against the

numerous species and serotypes of enteric bacteria. Thus, it would be useful to develop attenuated bacterial vaccine strains that are capable of inducing cross-protective immunity.

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SUMMARY OF THE INVENTION

The inventors have discovered that by combining, in a live attenuated derivative of an Enterobacteriaceae, a genetic construction that allows regulated expression of a regulatory protein such that antigenic proteins which are conserved among Enterobacteriaceae are expressed *in vivo*, and a means for regulatable synthesis of LPS O-antigens such that said O-antigens cease to be expressed *in vivo*, said live attenuated derivative has enhanced ability to induce cross-protective immunity against a diversity of gram negative pathogens. As used herein, the term "pathogen" refers to organisms that cause disease symptoms in an animal. A pathogen need not necessarily cause disease symptoms in the animal to which the live attenuated derivative is administered. For example, many *Salmonella* serotypes are not pathogens for chickens and swine, but persist commensally, and then become pathogens in humans when transferred through the food chain. Thus, the term pathogen as used herein would apply to such *Salmonella* serotypes.

The inventors have shown that the above described live attenuated derivatives are effective in colonizing in the intestinal tract of an individual and invading into lymphoid tissue such that a high-level immune response is induced which protects the individual from infection from a diversity of species or serotypes of bacterial pathogens. A further advantage of such a live attenuated derivative is that even when administered to an individual at exceedingly high doses, the risk of death is low.

In one embodiment of the invention, the regulatory protein is a ferric uptake regulator protein (Fur), which is encoded by the *fur* gene. The inventors have shown that by replacing the *fur* promoter with a regulatable promoter, the bacterial strain can be attenuated while still maintaining its immunogenicity. In a preferred embodiment of the invention, such regulated expression can be achieved by replacing the promoter for the *fur* gene with a metabolically controlled promoter such as that of the arabinose operon, the *araC*P_{BAD} activator-repressor-promoter system.

Synthesis of LPS O-antigen can be regulated by any means known in the art. For example, synthesis of O-antigen may be regulated by mutation of or regulation of any of the genes in the *rfb* gene cluster, or by mutation or regulation of RfaH or the JUMPstart sequence located upstream of the O-antigen gene cluster, or by mutation of or regulation of any of the other genes involved in regulation of any of the genes of the O-antigen gene cluster. (Iredell 1998; Wang 1998; Schnaitman 1993; Klena 1998; Kelly 1996). In one embodiment of the invention, synthesis of LPS O-antigen is regulated by means of a mutation in a *pmi* gene, which encodes phospho-mannose isomerase. Live attenuated derivatives harboring such a *pmi* mutation cannot synthesize LPS O-antigen side chains unless grown in the presence of free mannose. Thus, such mutants are unable to synthesize O-antigen side chains *in vivo*, as mannose in a free non-phosphorylated form is not prevalent in animal tissues. The presence of the *pmi* mutation leads to a gradual elimination of LPS O-antigen side chains *in vivo*, which then better exposes the LPS core and the IROMPs and other proteins involved in iron uptake, along with other surface proteins, which are conserved among genera and species within the Enterobacteriaceae family. Thus, the live attenuated derivative comprising the combination of the above described elements, when administered to an animal has enhanced ability to induce immune responses to IROMPs and other Fur regulated proteins and to the LPS core antigen to confer cross-protection against infection by diverse genera species and serotypes of Enterobacteriaceae.

Some embodiments of the invention may further comprise a means for decreasing the expression of antigenic proteins and carbohydrates that show a great degree of diversity

among the Enterobacteriaceae. These embodiments have the advantage of directing the immune response of the host animal to the conserved antigens, such that the cross-protective immunity is enhanced. Examples of such non-conserved antigenic proteins and carbohydrates include the flagella, LPS O-antigens, and fimbriae. In one embodiment, the *fliC* or *fljB* genes, which encode flagella are mutated. In another embodiment, both the *fliC* and *fljB* genes are mutated.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A and 1B illustrate the construction of a suicide vector for transfer of ΔPfur223::TT*araC*P_{BAD}*fur* deletion-insertion mutation.

FIG. 2 shows the ΔPfur223::TT*araC*P_{BAD}*fur* deletion-insertion chromosomal construction.

FIG. 3 illustrates the construction of a suicide vector for *pmi* deletion.

FIG. 4 shows the chromosomal deletion for Δ*pmi*-2426.

FIG. 5 demonstrates the reduction of LPS O-side chains in χ8650 as a function of time (hours) or numbers of generations of growth.

FIG. 6 demonstrates the outer membrane protein expression profile of ΔPfur223::TT *araC*P_{BAD}*fur* mutants grown in nutrient broth +/- arabinose.

FIG. 7 is a graphic illustration of colonization of Peyer's patches and spleens in 8-week-old female BALB/c mice as a function of time after oral inoculation with χ8634 ΔPfur::TT*araC*P_{BAD}*fur*.

FIG. 8 is a graphic illustration of colonization of Peyer's patches and spleens in 8-week-old female BALB/c mice as a function of time after oral inoculation with χ8650 Δ*pmi-2426*.

FIG. **9** is a graphic illustration of colonization of Peyer's patches and spleens in 8-week-old female BALB/c mice as a function of time after oral inoculation with χ8754 Δ*pmi-2426* ΔPfur223::*araC*P_{BAD}*fur*.

FIG. **10** illustrates the ability of χ8754, grown either in the presence or absence of mannose, to colonize the Peyer's patches and spleen of 8-week-old female BALB/c mice at designated intervals after oral inoculation.

FIG. **11** is a graphic illustration of the ability of serum antibodies collected from mice 30 days after oral inoculation with either χ8650 or χ8634 to react with the OMPs present in various Salmonella and E. coli strains grown in media containing excess iron such that the synthesis of IROMPs is minimal.

FIG. **12** is a graphic illustration of the ability of serum antibodies collected from mice 30 days after oral inoculation with either χ8650 or χ8634 to react with the IROMPS present in various Salmonella and E. coli strains grown in media substantially free of iron such that constitutive expression of fur-regulated proteins occurs.

FIG. **13** is a graphic illustration of colonization of day-of-hatch chicks as a function of time after oral inoculation with χ8754 Δ*pmi-2426* ΔPfur223::*araC*P_{BAD}*fur*.

FIG. **14** illustrates construction of the suicide vector for transfer of Δ*fliC825* deletion mutation.

FIG. **15** illustrates construction of a suicide vector for transfer of Δ*fljB217* deletion mutation.

FIG. **16** shows the Δ*fliC825* (A) and Δ*fljB217* (B) chromosomal deletion mutations.

DESCRIPTION OF THE INVENTION

The invention is directed to live attenuated strains of Enterobacteriaceae that are capable of inducing cross-protective immunity to a diversity of Enterobacteriaceae species and serotypes. This objective has been achieved by the means and methods described herein.

The Enterobacteria family comprises species from the following genera, any of which are considered to be useful in practicing the claimed invention: *Alterococcus, Aquamonas, Aranicola, Arsenophonus, Brenneria, Budvicia, Buttiauxella, Candidatus Phlomobacter, Cedeceae, Citrobacter, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Salmonella, Samsonia, Serratia, Shigella, Sodalis, Tatumella, Trabulsiella, Wigglesworthia, Xenorhbdus, Yersinia, Yokenella.* Due to their clinical significance, *Escherichia coli, Shigella, Edwardsiella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Morganella, Providencia* and *Yersinia* are considered to be particularly useful. Some embodiments of the instant invention comprise species of the *Salmonella* genera, as this genera has been widely and extensively studied and characterized.

The LPS of *Enterobacteriaceae* comprises three distinct domains: 1) the O-specific polysaccharide (O-antigen); 2) the core oligosaccharide (consisting of the inner and outer core oligosaccharides); and 3) the lipid A. LPS is both a major virulence factor and a target for protective immune responses. The core region of LPS is highly conserved, in contrast to the O-antigen which is the basis for distinguishing the various serotypes of many Enterobacteriaceae species. In *Salmonella*, for example, over 2,000 serotypes have been identified on the basis of the diversity of their O-antigen type and their flagella type. In contrast, those serotypes of *Salmonella* share only two closely related LPS core types.

The ability of Enterobacteriaceae to colonize the intestinal tract of animals is dependent upon, among other factors, the expression of a number of surface antigens, including LPS O-antigen side chains, a diversity of fimbrial adhesins, flagella and other outer membrane

proteins. LPS O-antigens are antigenically diverse as between strains of Enterobacteriaceae, and are a major factor in the variable immune response of host organisms to different strains of bacteria. It is known in the art that bacterial strains defective in the ability to synthesize LPS O-antigen substantially lack the ability to colonize the intestinal tract and to attach to and invade intestinal cells and survive in cells on the other side of the intestinal wall.

Thus, the bacterial strains of the invention comprise a means for regulatable synthesis of LPS O-antigens, such that O-antigens are synthesized when the strain is grown in vitro, and O-antigens cease to be synthesized in vivo, i.e., when the bacterial strains are administered to an animal. LPS O-antigen synthesis is dependent on a host of genes, including the genes of the *rfb* gene cluster. Regulation of synthesis of LPS O-antigens can be achieved by any suitable means. In some embodiments of the invention, regulation is achieved by mutations to or regulation of genes involved in synthesis of the O-antigens.

In some embodiments, the *pmi* gene is mutated such that the gene product is not expressed. The *pmi* gene encodes phosphomannose isomerase, a sugar transferase which inter converts mannose 6-phosphate and fructose 6-phosphate. In the process of O-antigen synthesis, mannose 6-phosphate is then converted to GDP-mannose which is then used for synthesis of O-antigen side chains. Thus, bacterial strains with a mutation which renders the *pmi* gene inoperable fail to produce O-antigen side chains. However, when such mutants are grown on media containing mannose, they are able to produce wild-type levels of O-antigen side chains. This is advantageous because of the important role that the LPS, including the O-antigen side chains, plays in the colonization of the gut and deep tissues of the animal. When the strain is administered to the animal, where free non-phosphorylated mannose is no longer available, the strain ceases to synthesize O-antigen side chain and over the course of several generations the strain no longer has significant levels of O-antigen associated with the cell wall, thus exposing the LPS core to enhance the immune response to this highly conserved antigen. Therefore, another advantage of the *pmi* gene mutation is that the mutation does not affect the ability of the strain to synthesize LPS core. Thus, the mutant strain can be grown on media containing mannose to maintain wild-type expression of O-antigen and then when administered to an

animal, will continue to express wild-type levels of LPS core while at the same time expression of the O-antigen side chains will be significantly diminished, resulting in enhanced immune response of the animal to the LPS core and diminished immune response to the O-antigen side chain.

Other means of regulating the synthesis of O-antigen side chains are expected to achieve the same advantages as described above with respect to the *pmi* mutation. Those of ordinary skill in the art will be able to devise other means of regulated synthesis of O-antigen side chains that meet the criteria of the invention based on the knowledge in the art of the process by which O-antigen is synthesized in Enterobacteriaceae. It is contemplated that those means are within the scope of the present invention. For example, the promoter for any of the *rfb* genes, which are needed for the synthesis of the LPS O-antigen, can be replaced with the *araC*P_{BAD} activator-repressor-promoter system so that expression of the particular *rfb* gene is dependant on the presence of arabinose supplied in media during growth of the vaccine.

The bacterial strains of the invention also comprise a genetic construction that allows regulated expression of a regulatory protein, such that antigenic proteins or carbohydrates which are conserved among the Enterobacteriaceae are expressed *in vivo*. Among the proteins or carbohydrates expressed in the cell membrane and wall of Enterobacteriaceae, some have been shown to be conserved to varying degrees among the various genera and species. For example, the LPS core and iron regulated outer membrane proteins (IROMPs) have been shown to be antigenically conserved among the Enterobacteriaceae.

IROMPS are encoded by a number of genes, the expression of which is controlled by a repressor protein (Fur) encoded by the *fur* gene. In the presence of iron, such as in the intestinal lumen, Fur represses the expression of IROMPs. In the absence of iron, such as for example in most animal host tissues beyond the intestinal wall barrier, Fur repression ceases, and thus IROMPs and other Fur-regulated genes are highly expressed. While *fur* mutants have been shown to be attenuated when administered orally to animals, such *fur* mutants may be susceptible to iron toxicity in the intestinal lumen due to non absorption of dietary iron and the

presence of iron from hemoglobin breakdown contributed into the intestinal tract as a component of bile. In addition, unless in a complex form, iron can promote the formation of damaging hydroxyl radicals, which may account in part for the toxicity of iron. Further, since *fur* has been shown to play a role in the acid tolerance of Enterobacteriaceae, *fur* mutants are potentially sensitive to the gastric acidity barrier and to killing in acidified phagosomes in macrophages. All of these factors contribute to the fact that while *fur* mutants would display high levels of IROMPs that induce cross protective immunity, the avirulence properties of such mutants make them poor immunogens.

Thus, some embodiments of the bacterial strains of the present invention comprise a genetic construction which allows for regulated expression of the *fur* gene, such that *fur* is expressed when the strain is grown *in vitro*, and in the intestinal lumen, but is not expressed when the bacterial strain is in the host tissue beyond the intestinal wall barrier. Thus, the bacterial strain exhibits wild-type repressed levels of IROMP expression during growth *in vitro* and during the initial stage of infection, i.e. when in the intestinal lumen. Then after colonization of the lymphoid organs beyond the intestinal wall barrier, the strain exhibits constitutive expression of IROMPs and other Fur-regulated proteins.

The regulated expression of the gene encoding a regulatory protein may be achieved by any means available in the art. For example, it is common practice to delete the wild type promoter associated with a particular gene and replace it with a promoter from the same or a different organism that is regulatable. In one embodiment of the present invention, the genetic construction is one in which expression of the *fur* gene is dependent upon the presence of arabinose. Arabinose can be supplied in culture media, and is also present in the intestinal tract of animals, as a component of plants which constitute a common part of animal diets. However, arabinose is not present in animal tissues beyond the intestinal wall barrier. This is achieved by replacing the *fur* promoter with the *araC*P_{BAD} activator-repressor-promoter system. The *araC*P_{BAD} activator-repressor-promoter is dependent on the presence of arabinose, which binds to the *araC* gene product to activate transcription from the P_{BAD} promoter. So, when the *araC*P_{BAD} activator-repressor-promoter is operatively linked to the *fur* gene, in place of the *fur* promoter,

expression of the *fur* gene is then dependent on the presence or absence of arabinose. For example, when the bacterial strain harboring such a genetic construction is grown in media supplemented with arabinose, or alternatively when the strain is in the lumen of the intestinal tract of an animal where arabinose is present, the *fur* gene is expressed and the expression IROMPs and other *fur* regulated proteins is repressed. On the other hand, when such a bacterial strain invades the tissue on the other side of the intestinal wall barrier, where arabinose is absent, the *fur* gene is no longer expressed leading to high level of expression of all of the *fur* regulated proteins including IROMPs.

Some embodiments of the bacterial strains of the invention comprise mutations in genes that encode other antigenic proteins expressed on the surface of *Enterobacteriaceae*, but which proteins are not antigenically conserved among the genera and species of the *Enterobacteriaceae* family. Such mutations cause diminished expression of those proteins, such that the host immune response is focused on the conserved antigenic proteins and carbohydrate antigens, further enhancing cross-protective immunity. It is important that such mutations be selected such that the diminished expression of the particular gene product does not significantly inhibit the bacterial strain's ability to colonize the intestinal tract and invade and persist in the tissue beyond the intestinal wall barrier. Examples of other surface proteins that are not antigenically conserved among the *Enterobacteriaceae* include flagella, pili, and fimbriae among others. Some embodiments of the bacterial strains of the invention comprise genetic constructions that diminish the expression of flagella. In particular embodiments, the bacterial strains comprise mutations in the *fliC* or *flgB* genes, or both the *fliC* and *flgB* genes. Such mutations do not alter the ability of the bacterial strains to colonize the mucosal tissue of the animal or invade and persist in the tissue beyond the lumen of the intestine. It is expected, since the flagella are antigenically diverse among the Enterobacteriaceae, that such mutations will enhance the cross-protective immunity elicited by such strains upon administration to animals. The skilled artisan will appreciate that diminished expression of other surface proteins that are antigenically diverse will confer similar characteristics as described with respect to the *fliC* and *flgB* mutations, thus achieving the same advantages as those mutations.

In a particular embodiment, the bacterial strains of the invention comprise a mutation in the *pmi* gene which renders that gene inoperable. A particularly preferred embodiment comprises the Δ*pmi-2426* mutation, which is described below in the Examples. The strain further comprises a genetic construction wherein the native *fur* gene promoter has been replaced by the *araC*P_{BAD} activator-repressor-promoter system. A particularly preferred embodiment comprises the ΔPfur223::TT *araC*P_{BAD} *fur* construction. A particularly preferred bacterial strain, which comprises the above mentioned genetic constructs is χ8754, the construction of which is described in detail in the Examples. The χ8754 strain exhibits wild-type levels of LPS O-antigen and wild-type repressed levels of IROMPs both during growth of the strain and during initial stages of infection of visceral organs whether administered orally or by course spray to young chickens. Then after colonization of visceral lymphoid organs, LPS O-antigen synthesis ceases and overexpression of IROMPs commences. Thus, this strain is attenuated, efficiently colonizes lymphoid tissues following oral administration to animals and induces high-level protective immunity to subsequent challenge with a plurality of wild-type *Enterobacteriaceae*.

In an alternative of the embodiment described immediately above, instead of mutating the *pmi* gene, the *pmi* promoter is replaced with the *araC*P_{BAD} activator-promoter. Thus, only after several generations of growth *in vivo* would LPS O-antigen cease.

The invention further comprises methods for inducing an immune response comprising administering any of the above described bacterial strains to an animal. Such bacterial strains may be administered by any means known in the art. Preferred methods of administration include, for example, oral administration, gastric intubation, or in the form of aerosols, for example by the whole-body spray method described in PCT publication WO 00/04920. Other methods of administration are also possible, for example by injection. Dosages required for induction of cross-protective immunity will vary, although routine experimentation will allow the skilled artisan to make such determinations. Pharmaceutical carriers, in which the bacterial strains are suspended are also known in the art.

Administration of the bacterial strains of the invention can be a singl dose, or as is not uncommon, in a series of two or more doses. Such subsequent administrations of the bacterial strain are commonly referred to as boosters, and in many instances such boosters result in prolonged protection of the host animal.

The above disclosure describes several embodiments of the invention, and the examples below further illustrate the invention. The skilled artisan will recognize that other embodiments that provide the same advantages may also be employed in the practice of this invention. The scope of this invention is intended to be defined by the claims, and the description and examples are intended to be non-limiting.

EXAMPLES

Table 1 lists the bacterial strains referred to throughout the Description and Examples, and Table 2 lists the plasmids used in the following Examples.

Table 1. Bacterial Strains

Strain #	Strain	Phenotype/Genotype or	Reference/Source
A. Escherichia coli			
DH5α	*E. coli* K-12	Δ(*lacZYA-arg F*)*U169* (ϕ80 *lacZ* ΔM15) *glnV44 recA1 endA1 gyrA96 thi-1 relA1 hsdR17*	1
MGN-617	*E. coli* K-12	SM10 λ*pir* Δ*asdA4* Δ*zhf-2*::Tn*10*	2
χ289	*E. coli* K-12	F- prototroph	3
χ6206	*E. coli* 026:H11	EPEC	S. Ashkenazi
χ6212	*E. coli* K-12	Δ*asdA4* Δ*zhf-2*::Tn*10* derivative	DH5λ
χ7122	Avian *E. coli*	O78:K80:H9	4
χ7235	Avian *E. coli* TK3	O1:K1:H7	5
χ7302	Avian *E. coli* MT512	O2:K1:H+	6
B. Salmonella enterica			
χ3201	*S. agona* NR1	wild-type group B (1,4,12)	7
χ3202	*S. albany* NR2	wild-type group C_3 (8,20)	7
χ3203	*S. anatum* NR3	wild-type group E_1 (3,10)	7
χ3206	*S. bredeney* NR8	wild-type group B (1,4,12,27)	7
χ3210	*S. hadar* NR14	wild-type group C_2 (6,8)	7
χ3212	*S. heidelberg* NR99	wild-type group B (1,4,5,12)	7
χ3213	*S. infantis* NR29	wild-type group C_1 (6,7)	7
χ3217	*S. montevideo* NR35	wild-type group C_1 (6,7)	7
χ3220	*S. panama* NR38	wild-type group D (1,9.12)	7
χ3246	S. choleraesuis	wild-type group C_1 (6,7)	8
χ3339	S. typhimurium *SL1344*	*hisG46*	9
χ3700	*S. enteritidis* 4973	wild-type group D (1,9,12) PT13A	7
χ3744	*S. typhi* ISP1820	wild-type group D (9,12)	10
χ3761	*S. typhimurium* UK-1	wild-type group B (1,4,12)	11
χ3796	*S. gallinarum*	wild-type group D (1,9,12)	C. Poppe
χ3847	*S. enteritidis* Y-8P2	wild-type group D (1,9,12) PT8	7
χ3848	*S. enteritidis* 27A	wild-type group D (1,9,12) PT8	7
χ3850	*S. enteritidis* B6996	wild-type group D (1,9,12) PT13A	7
χ3851	S. enteritidis	wild-type group D (1,9,12) PT4	Curtiss Collection
χ3985	*S. typhimurium* UK-1	Δ*cya-12* Δ*crp-11*	11
χ4235	*S. kentucky*	wild-type group C_3 (8,20)	Curtiss Collection

Table 1. Bacterial Strains

Strain #	Strain	Phenotype/Genotype or	Refer nce/Sour ce
χ4433	*S. typhimurium* F98	wild-type group B (1,4,12)	7
χ4860	*S. dublin*	wild-type group D (1,9,12)	C. Maddox
χ4971	*S. typhimurium* UK-1	fur-1	12
χ8387	S. paratyphi *A*	cryptic plasmid cured	Curtiss Collection
χ8407	S. muenster	wild-type group E_1 (3,10)	Curtiss Collection
χ8409	S. senftenberg	wild-type group E_4 (1,3,19)	Curtiss Collection
χ8438	*S. typhi* Ty2	*Cys, rpoS*$^+$ group D (9,12)	13
χ8634	*S. typhimurium* UK-1	ΔPfur223::TT *araC* P_{BAD}*fur*	This application
χ8650	*S. typhimurium* UK-1	Δ*pmi-2426*	This application
χ8754	*S. typhimurium* UK-1	Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur*	This application
χ8600	*S. typhimurium* SL1344	Δ*fliC825 hisG46*	χ3339
χ8601	*S. typhimurium* SL1344	Δ*flgB217 hisG46*	χ3339

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Table 2. Plasmids

Plasmids	Description	Derivation/source
pCR-Blunt II	TOPO vector	Invitrogen
pDMS197	SacB suicide vector	Curtiss collection
pRE112	SacB suicide vector	Curtiss collection
pMEG-208	Asd^+ vector with TT *araC* P_{BAD}	Megan Health, Inc
pMEG-375	SacB SacR Pir-dependent suicide vector	Megan Health, Inc
pMEG-855	Suicide vector for ΔPfur223::TT *araC*P_{BAD}*fur*	Megan Health, Inc
pYA3546	Suicide vector for Δ*pmi-2426*	Curtiss collection
pYA3547	Suicide vector for Δ*fliC825*	Curtiss collection
pYA3548	Suicide vector for Δ*fljB217*	Curtiss collection

Example 1. Construction of a bacterial strain with arabinose-dependant regulation of the *fur* gene which in turn regulates expressi n of numer us genes enabling uptake f ir n by bacterial cells.

S. typhimurium fur mutants are completely attenuated for mice and chickens but are not very immunogenic. This is undoubtedly due to the fact that *fur* mutants constitutively express a diversity of genes resulting in very efficient uptake of iron that is quite prevalent in the intestinal tract due to dietary non-absorption of iron and due to the presence of iron as a breakdown product of hemoglobin secreted in bile into the duodenal contents of the intestine. Since high intracellular iron concentrations are toxic to bacteria, *fur* mutants do not survive very well in the intestinal tract and therefore are not very efficient in colonization of the GALT, which is necessary in order to be immunogenic. One way to circumvent this problem would be to have the *fur* gene expressed when the bacterial cells are present in the intestinal contents so that efficient colonization of the GALT can take place followed by the gradual cessation in synthesis of the *fur* gene product *in vivo* to result in an attenuated phenotype. In addition, the gradual constitutive expression of *fur* regulated genes would expose the immunized animal host to over expression of iron regulated outer membrane protein (IROMP) antigens as well as other proteins involved in the acquisition, transport and delivery of iron to the bacterial cells. Since many *fur* regulated gene products are closely related structurally among Gram-negative bacterial species, antibodies induced in an immunized animal host to the IROMPs and other *fur* regulated gene products of one bacterial species react with the homologous proteins expressed by other Gram-negative bacterial pathogens. It should be emphasized that synthesis of *fur* regulated gene products *in vivo* is essential for virulence since a major host defense mechanism is to sequester iron via transferrin, lactoferrin and other iron binding proteins so as to make iron unavailable to invading bacterial pathogens. Thus, antibody responses to these proteins are often protective in preventing successful infection of bacterial pathogens that succeed by *in vivo* multiplication. A corollary is that induction of high-level immune responses to the IROMPs and other *fur* regulated gene products is quite effective in inducing antibodies that are cross protective and prevent infection of an immunized animal host by a diversity of Gram-negative bacterial pathogens.

One means to achieve regulated expression of the *fur* gene is to replace the promoter for the *fur* gene, whose function is regulated by both iron concentration and glucose concentration via the process of catabolite repression, with a metabolically controlled promoter such as that of the arabinose operon. The *araC* P_{BAD} activator-promoter is dependent on the presence of arabinose that binds to the *araC* gene product to activate transcription from the P_{BAD} promoter. Thus, if the *araC* P_{BAD} activator-promoter is used to replace the *fur* promoter and the structural gene for the *fur* gene left intact, expression of the *fur* gene will be dependent on the presence or absence of arabinose. Since arabinose is quite prevalent in plants, some free arabinose exists in the diets consumed by many animals and humans thus contributing to the continued expression of a *fur* gene operationally linked to the *araC* P_{BAD} activator-promoter while bacteria remain in the intestinal tract. On the other hand, arabinose is absent in animal tissues and the *fur* gene product will cease to be synthesized and will thus be diluted out as a consequence of bacterial cell division. Thus, after several cell divisions, constitutive expression of *fur* regulated genes will commence leading to attenuation, on the one hand, and exposure of the immunized animal host to all the *fur* regulated protein antigens, on the other.

To achieve these objectives, primers 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) (Figure 1-A) were used to PCR amplify a 545 bp fragment from the chromosome of *S. typhimurium* UK-1 χ3761 containing 321 bp upstream of the *fur* gene and 224 bp of the *fur* gene. This blunt-ended PCR amplified DNA fragment was cloned by blunt-end ligation into the pCR-BluntII-TOPO vector (Figure 1-A, Table 2) which is designed to facilitate blunt-end ligation. The resulting plasmid pMEG-840 (Figure 1-A) was subjected to an inverse PCR reaction using primers 3 (SEQ ID NO:3) and 4 (SEQ ID NO:4) (Figure 1-A) to delete 140 bp containing the *fur* gene promoter from 161 to 22 bp upstream of the *fur* gene ATG start site. The product of this reaction was subjected to blunt-end ligation to yield pMEG-853 (Figure 1-A). The ΔPfur mutation of 140 bp possessed internal restriction sites for *Bgl*II and *Nhe*I separated by 4 bp that would permit insertion of the *araC* P_{BAD} activator-promoter. pMEG-853 was digested with *Spe*I and *Eco*RV and the 472 bp fragment containing the ΔPfur mutation was cloned into the suicide vector pRE112 (Figure 1-A; Table 2) that had been digested with *Xba*I and *Sma*I enzymes to yield pMEG-854 (Figure 1-A; 1-B). It should be noted that the restriction enzymes *Spe*I and *Xba*I

generate the same CTAG internal overlapping sticky ends and both *Eco*RV and *Sma*I generate blunt ended sequences to enable success in the cloning and ligation of the 472 bp sequence from pMEG-853 cloned into pRE112 to yield pMEG-854. pMEG-854 contains a 405 bp fragment containing a sequence upstream of the *fur* gene promoter fused to a sequence encompassing the Shine-Dalgarno sequence and beginning of the *fur* gene, which thus contains the ΔPfur mutation. Oligonucleotide primers 5 (SEQ ID NO:5) and 6 (SEQ ID NO:6) (Figure 1-B) were used to PCR amplify the sequence from pMEG-208 (Figure 1-B) containing a transcription terminator (TT) and the *araC* P_{BAD} activator-promoter. This DNA fragment contains a *Bgl*II site and an *Xba*I site encoded in primer 6 (see Figure 1). Since the *Xba*I site generates a CTAG overhang, it is hybridizable with DNA fragments cut with the *Nhe*I restriction enzyme that also generates a CTAG hybridizable sequence. The PCR amplified TT *araC* P_{BAD} fragment from pMEG-208 was therefore digested with *Bgl*II and *Xba*I and cloned into pMEG-854 digested with *Bgl*II and *Nhe*I to yield the suicide vector pMEG-855 (Figure 1-B).

pMEG-855 was transferred to the suicide vector donor strain MGN-617 (Table 1) that was mated with χ3761 (Table 1). Chloramphenicol-resistant transconjugants that had inherited the suicide vector into the chromosome by a single crossover event were selected by plating on L agar containing chloramphenicol. Ten recombinant colonies were selected and purified on L agar medium with chloramphenicol and individual colonies picked into 1.0 ml of L broth lacking chloramphenicol and incubated at 37°C. Following growth to approximately 10^8 CFU, sucrose-resistant isolates were obtained by plating on CAS plates containing 5 % sucrose but lacking arabinose. This procedure is selective for a second crossover event in which the wild-type *fur* promoter would be replaced with the TT *araC* P_{BAD} activator-promoter that would cause *fur* gene expression to be dependent on the presence of arabinose. Colonies containing cells lacking the ability to synthesize the *fur* gene product have a 3 to 4 mm orange halo surrounding colonies whereas this orange halo is only 1 mm when cells are plated on CAS medium containing 0.2% arabinose. The ΔPfur223::TT *araC* P_{BAD} *fur* construction present in the stocked strain χ8634 is diagramed in Figure 2.

Example 2. Generation of a defined deletion mutation in the *pmi* gene and construction of *Salmonella typhimurium* mutants with this Δ*pmi-2426* mutation.

An 1881 bp *S. typhimurium* DNA sequence encompassing the *pmi* gene was PCR amplified from the S. typhimurium UK-1 χ3761 chromosome. As depicted in Figure 3, oligonucleotide primers 7 (SEQ ID NO:7) and 8 (SEQ ID NO:8) were designed to amplify the 298 bp sequence 5′ to the ATG start codon of the *pmi* gene to yield the N-flanking fragment. Similarly, oligonucleotide primers 9 (SEQ ID NO:9) and 10 (SEQ ID NO:10) were designed to amplify the 301 bp sequence 3′ to the TAG stop codon of the *pmi* gene to yield the C-flanking fragment. The N-flanking and C-flanking DNA fragments (Figure 3) were then digested with *Eco*RI, ligated with polynucleotide joining enzyme after which oligonucleotide primers 7 and 10 were used to amplify the ligated N-flanking and C-flanking fragments by PCR. The PCR amplified oligonucleotide was then digested to completion with *Kpn*I and *Sac*I and cloned into the suicide vector pMDS197 (Table 2) similarly digested with *Kpn*I and *Sac*I. The resulting recombinant suicide vector, pY3546, is depicted in Figure 3. This suicide vector contains the N-flanking and C-flanking sequences adjacent to the *pmi* gene, which has been deleted with the 1176 base pair *pmi* gene replaced with an *Eco*RI recognition sequence.

The suicide vector pYA3546 was introduced by electroporation into the suicide vector donor strain MGN-617 (Table 1). This recombinant strain was then mated with the *S. typhimurium* UK-1 strain χ3761 (Table 1) and tetracycline-resistant transconjugants were selected that arose due to single cross over events integrating pYA3546 into the chromosome of χ3761. Ten tetracycline-resistant transconjugants were selected, purified by restreaking on tetracycline-containing medium and grown in tetracycline-free Luria broth as 1 ml cultures to an approximate density of 10^8 CFU/ml. These cultures were plated in the presence of 5% sucrose to select for a second crossover event to excise the suicide vector from the chromosome but leave in its place the deletion of 1176 bp encoding the *pmi* gene. Individual isolates were tested for inability to ferment mannose on MacConkey-Mannose agar and one isolate designated χ8650 was stocked and the *pmi* allele designated *pmi-2426*. The chromosomal Δ*pmi-2426* mutation

present in χ8650 is diagramed in Figure 4 along with the genes flanking the deleted *pmi* mutation in the *S. typhimurium* chromosome.

Example 3. Introduction of Δ*pmi-2426* mutation into χ8634.

The suicide vector pYA3546 (Figure 3) for introduction of the Δ*pmi-2426* mutation by allele replacement was introduced into MGN-617 (Table 1) and this strain mated with χ8634 possessing the ΔPfur223::TT *araC* P_{BAD}*fur* mutation. Tetracycline-resistant transconjugants were selected on L agar medium containing tetracycline and 0.2% arabinose. It should be noted, that strains with the ΔPfur223::TT *araC* P_{BAD}*fur* mutation grow rather poorly on medium without any added arabinose. Ten tetracycline-resistant transconjugants were purified by restreaking on L agar medium containing tetracycline and 0.2% arabinose. Individual colonies were picked into 1.0 ml of L broth containing 0.2% arabinose. When cultures reached approximately 1 x 10^8 CFU, sucrose-resistant isolates, in which a second crossover event had occurred, were selected by plating on L agar medium containing 5% sucrose and 0.2% arabinose. Sucrose-resistant isolates were picked and tested for sensitivity to tetracycline indicating loss of the suicide vector and for inability to ferment mannose by streaking on MacConkey-Mannose agar. One isolate having all of the correct phenotypic properties with regard to the presence of the Δ*pmi-2426* and ΔPfur223::TT *araC* P_{BAD}*fur* mutations was stocked as χ8754.

Example 4. Phenotypic properties of χ8634, χ8650 and χ8754.

χ8634 with the ΔPfur223::TT *araC* P_{BAD}*fur* mutation, χ8650 with the Δ*pmi-2426* mutation and χ8754 with both mutations were compared to the wild-type *S. typhimurium* UK-1 strain χ3761 for ability to ferment various carbohydrates contained at a 0.5% concentration in MacConkey agar. As indicated by the data in Table 3, all strains are unable to ferment lactose whereas χ8650 and χ8754 are unable to ferment mannose. All other sugars were fermented by all four strains.

Table 3. Carbohydrate fermentations[a]

Strains/genotype	Carbohydrates							
	Lac	Glc	Man	Mal	Srl	Xyl	Ara	Fru
χ3761 wild-type	-	+	+	+	+	+	+	+
χ8634 ΔPfur223::TT *araC* P_{BAD}*fur*	-	+	+	+	+	+	+	+
χ8650 Δ*pmi-2426*	-	+	-	+	+	+	+	+
χ8754 Δ*pmi-2426* Δfur223::TT *araC* P_{BAD}*fur*	-	+	-	+	+	+	+	+

[a] Bacterial strains were grown in L broth at 37°C overnight and the cultures streaked to observe isolated colonies on MacConkey agar with 0.5% each of the sugars indicated. Plates were incubated overnight. Lac, lactose; Glc, glucose; Man, mannose; Mal, maltose; Srl, sorbitol; Xyl, xylose; Ara, arabinose; Fru, fructose; -, no fermentation; +, fermentation.

The same four strains were evaluated for production of the group B LPS O-antigen side chains and for presence of flagellar antigens using slide agglutination assays with antisera obtained from Difco Laboratories. The results presented in Table 4 are as expected. It should be noted that L agar, which contains yeast extract, contains a low concentration of mannose. Thus strains with the Δ*pmi-2426* mutation when grown in L broth or on L agar make a lower than usual level of O-antigen side chains than if grown in medium with added mannose but a higher amount than when grown in a medium totally devoid of mannose. For example, if the strains are grown in Nutrient broth or on Nutrient agar medium without added mannose, the amount of O-antigen side chains synthesized is very negligible as revealed by resistance of the strains to infection with bacteriophage P22 whose attachment to *S. typhimurium* is dependent on the presence of O-antigen side chains.

Table 4. Slide agglutination assays with *Salmonella* O and H anti-sera[a]

Strains/genotype	Group B O antiserum factors 1, 4, 5,12	H antiserum polyA

Table 4. Slide agglutination assays with *Salmonella* O and H anti-sera[a]

Strains/genotype	Group B O antiserum factors 1, 4, 5,12	H antiserum polyA
□3761 wild-type	+++	+++
□8634 ΔPfur223::TT *araC* P_{BAD}*fur*	+++	+++
□8650 Δ*pmi-2426*	++	+++
□8754 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur*	++	+++

[a] Bacterial strains were grown on L agar without mannose and arabinose. A single colony of each of the strains was picked and suspended in buffered saline with gelatin (BSG) on a microscope slide, and mixed with 5 µl of the anti-serum. Agglutination reactions were observed and compared. ++- moderate agglutination; +++- high agglutination.

Figure 5 presents the results of an experiment with χ8650 with the Δ*pmi-2426* mutation, which demonstrates that as a function of time or number of generations of growth in Nutrient broth medium in the absence of added mannose there is a gradual loss of LPS O-antigen side chains. This behavior is as expected and would be reproduced in vivo when a vaccine strain, after immunization of an animal host, enters visceral tissues which lack free non-phosphorylated mannose.

Based on the nature of mutational changes in χ8634 and χ8754, which both possess the ΔPfur223::*araC* P_{BAD} *fur* mutation, synthesis of IROMPs should be constitutive when those strains are grown in the absence of arabinose and absent when grown in the presence of arabinose. The synthesis of IROMPs should be unaffected by the presence or absence of arabinose during growth of χ3761 with the level of IROMPs dependant on the iron concentration in Nutrient broth. These predictions were evaluated by preparing overnight cultures of χ3761, χ8634 and χ8754 growing statically in 10 ml of Nutrient broth containing 0.2% arabinose at 37°C. The cultures were then diluted 1:1000 into 10 ml of prewarmed Nutrient broth with and without 0.2% arabinose and grown with aeration to a cell density of about 8 x 10^8 CFU/ml. The cultures were centrifuged at 5000 rpm at 4°C for 15 min in a refrigerated Sorvall clinical

centrifuge and the cell pellets suspended in 10 mM HEPES buffer. The bacterial suspensions were lysed by sonication with six 10 s pulses at 40 w. The sonicated suspensions were centrifuged at 15,600 rpm for 2 min at 4°C. The supernatant fluid was centrifuged again at 15,600 rpm for 30 min at 4°C. The cell membrane pellets were suspended in HEPES buffer and an equal volume of 2 % Sarkosyl added. The suspension was incubated at room temperature for 30 min with gentile shaking. Next, the outer membrane aggregate was sedimented by centrifugation at 15,600 rpm for 30 min at 4°C and the supernatant was discarded. The membrane pellets were washed with and re-suspended in HEPES buffer. The samples were prepared for the SDS-PAGE analysis by adding equal amounts of 2X sample buffer and boiling the samples for 10 min. Lastly, the samples were centrifuged at 12,000 rpm for I min in a microfuge and loaded onto gels containing SDS and 10 % polyacrylamide. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue. The results are depicted in Figure 6 and give the expected results based on the strain genotypes.

Example 5. Ability of mutant strains to colonize lymphoid tissues in mice.

The ability of *S. typhimurium* χ8634 with the ΔPfur223::*araC* P_{BAD} *fur* mutation to colonize eight-week-old female BALB/c mice following oral inoculation of 10^9 CFU was investigated. The bacteria were grown in Luria broth containing 0.2% arabinose to an OD_{600} of approximately 0.8. Bacteria were sedimented by centrifugation and concentrated by suspension in buffered saline with gelatin (BSG) so that 20 µl would contain approximately 10^9 CFU of bacteria. Groups of immunized mice were euthanized as a function of time after oral inoculation and the data pertaining to colonization of Peyer's patches and spleens are depicted in Figure 7. It is evident that χ8634 is quite effective in colonization of lymphoid tissues whereas a strain with a deletion of the *fur* gene colonizes tissues at very much lower titers such that animals do not develop immunity to subsequent challenge with virulent wild-type *S. typhimurium*. Results from an experiment done the same way for the *S. typhimurium* strain χ8650 with the Δ*pmi-2426* mutation are presented in Figure 8. In this case, bacteria were grown in Luria-Bertani broth with

or without 0.5% mannose prior to inoculation into mice. There were no significant differences for the two growth conditions.

Results of two other experiments with the *S. typhimurium* χ8754 strain that possesses both the ΔPfur223::TT *araC* P_{BAD} *fur* and Δ*pmi-2426* mutations are represented in Figures 9 and 10. It is evident that χ8754 persists for a sufficient time in lymphoid tissues to induce immunity before almost disappearing by 42 days (Figure 9). Results were not significantly different depending upon whether the cultures were grown in the presence or absence of mannose and arabinose prior to inoculation (Figure 10). This result is anticipated in that Luria broth, as indicated above, contains yeast extract that possesses both free arabinose and free mannose at low concentrations. When strains are grown in Nutrient broth, the differences are magnified but growth of *Salmonella* vaccine strains in Nutrient broth leads to a lesser degree of colonization and a lower immunogenicity. Growth in Nutrient broth is thus not a preferred method of evaluation for attenuated live vaccines.

Example 6. Avirulence and immunogenicity of *S. typhimurium* strains with Δ*pmi-2426* and/or ΔPfur223::TT *araC* P_{BAD} *fur* mutations.

Table 5 presents results of an experiment to evaluate the attenuation and immunogenicity of χ8634 with the ΔPfur223::TT *araC* P_{BAD} *fur* mutation. χ8634 was grown in Luria broth either without or with 0.2% arabinose to an OD_{600} of about 0.8. Bacterial cells were sedimented by centrifugation and suspended in BSG to a density so that there would be about 1 x 10^9 CFU in a 20 μl sample. Female BALB/c mice were purchased at 7 weeks of age and maintained for one week in our animal facilities to acclimate prior to use in experiments. At eight weeks of age, food and water were removed for four hours prior to oral inoculation with 20 μl of χ8634 cells suspended in BSG at appropriate densities. Morbidity and mortality were observed for 30 days, after which, survivors were challenged with virulent wild-type *S. typhimurium* UK-1 χ3761 grown in Luria broth to an OD_{600} of approximately 0.8. It is apparent from the results that growth in Luria broth without added arabinose conferred total avirulence and induced the highest

level of protective immunity. Since Luria broth contains yeast extract, which contains arabinose, it is evident that addition of an extra 0.2% arabinose must cause synthesis of too much Fur protein such that the total repression of all *fur*-regulated genes must starve cells for iron so that they are less able to survive and colonize in the intestine and thus are less immunogenic. This result has been observed in other experiments and thus growth of strains in Luria broth without added arabinose will be preferred to optimize immunogenicity. If, on the other hand, χ8634 is grown in Nutrient broth, which lacks arabinose, the addition of arabinose to 0.1 or 0.2% is necessary to achieve good immunogenicity.

Table 5. Virulence and protection of *S. typhimurium* UK-1 ΔPfur223::TT *araC* P_{BAD}*fur* mutant □8634 in 8-week-old female BALB/c mice following oral inoculation[a]

Growth condition	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
Luria broth	1.4×10^9	4/4	1.4×10^9	4/4
	1.4×10^8	4/4	1.4×10^9	4/4
	1.4×10^7	4/4	1.4×10^9	4/4
	1.4×10^6	4/4	1.4×10^9	3/4
	1.4×10^5	4/4	1.4×10^9	2/4
(Total)		20/20		17/20
Luria broth with 0.2% arabinose	1.1×10^9	4/4	1.4×10^9	4/4
	1.1×10^7	3/4	1.4×10^9	2/3
	1.1×10^6	4/4	1.4×10^9	1/4
	1.1×10^5	4/4	1.4×10^9	0/4
(Total)		15/16		7/15

[a] Bacteria were grown in Luria broth with or without 0.2% arabinose to OD_{600} of ~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 μl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 □3761 grown in

Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

To evaluate the attenuation and immunogenicity of *S. typhimurium* χ8650 possessing the Δ*pmi-2426* mutation, bacteria were grown in Nutrient broth with or without 0.5% mannose and 0.5% glucose to an OD_{600} of approximately 0.8. Bacterial cells were collected by centrifugation and suspended in a concentrated form in BSG so that a 20 μl sample would possess approximately 1 x 10^9 CFU. Female BALB/c mice were purchased at 7 weeks of age and maintained for one week in our animal facilities to acclimate prior to use in experiments. At eight weeks of age, food and water were removed for four hours prior to oral inoculation with χ8650 cells suspended in BSG at appropriate densities. Morbidity and mortality were observed for 30 days, after which, survivors were challenged with virulent wild-type *S. typhimurium* UK-1 χ3761 grown in Luria broth to an OD_{600} of approximately 0.8. It should be noted that the vaccine strain was grown in Nutrient broth since it is almost devoid of mannose to determine the influence of O-antigen side chain synthesis on the initial invasiveness of the candidate vaccine strain. On the other hand, we have demonstrated in many past studies that growth in Luria broth leads to optimal expression of the phenotype that is conducive to attachment to and invasion into the GALT of both virulent as well as of attenuated *Salmonella* vaccine strains. The results of this experiment are presented in Table 6. It is evident that growth of the vaccine strain under conditions that enable synthesis of LPS O-antigen side chains leads to morbidity and mortality at high doses (i.e., 1.5 x 10^9 CFU). However, mice that survived these high doses without morbidity, acquired protective immunity to high doses of the challenge strain. χ8650 grown in medium to preclude synthesis of LPS O-antigen side chains were totally attenuated and induced a high level of protective immunity (Table 6).

Table 6. Virulence and protection of *S. typhimurium* UK-1 Δ*pmi-2426* mutant □8650 in 8-week-old female BALB/c mice following oral inoculation[a]

Growth condition	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge

Table 6. Virulence and protection of *S. typhimurium* UK-1 Δ*pmi-2426* mutant □8650 in 8-week-old female BALB/c mice f llowing oral inoculati n[a]

Growth condition	Inoculating dose	Survivors/ total	Challenge dose	Survivors/tot al after challenge
Nutrient Broth + 0.5% Man + 0.5% Glc	1.5×10^9	3/8	8.0×10^8	3/3
	1.5×10^8	7/8[b]	8.0×10^8	4/4
	1.5×10^7	7/8	8.0×10^8	3/4
	8.0×10^7	3/3		
	1.5×10^6	4/4	8.0×10^7	4/4
	1.5×10^5	4/4	8.0×10^7	4/4
		(25/32)		**(21/22)**
Nutrient Broth:	1.7×10^9	8/8	8.0×10^8	4/4
	8.0×10^7	4/4		
	1.7×10^8	8/8	8.0×10^8	4/4
	8.0×10^7	4/4		
	1.7×10^7	7/8	8.0×10^8	3/3
	8.0×10^7	4/4		
	1.7×10^6	4/4	8.0×10^7	4/4
	1.7×10^5	4/4	8.0×10^7	2/4
		(31/32)		**(28/31)**

[a] Bacteria were grown in Nutrient broth with or without 0.5% mannose and 0.5% glucose to OD_{600} of ~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 □3761 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

[b] Three of the seven surviving mice (in one cage) appeared sick with loss of hair and were therefore not challenged.

We next investigated the attenuation and immunogenicity of χ8754, which possesses both the ΔPfur223::TT *araC* P_{BAD}*fur* and Δ*pmi-2426* mutations. χ8754 was grown in Luria broth supplemented with 0.5% mannose and 0.2% arabinose to an OD_{600} of approximately 0.8. Bacterial cells were concentrated by centrifugation and suspended in BSG such that a 20 µl inoculum would contain approximately 1 x 10^9 CFU. Eight-week-old female BALB/c mice that had been acclimated for a week were orally inoculated with 20 µl of inocula containing differing densities of χ8754 cells. All mice survived for 30 days as indicated by the results presented in Table 7. The surviving mice were challenged with 1.0 x 10^9 CFU of the wild-type virulent *S. typhimurium* UK-1 strain χ3761 and all but one mouse survived the challenge. In that we had found that χ8634 with the ΔPfur223::TT *araC* P_{BAD}*fur* mutation displayed total attenuation and highest immunogenicity when grown in Luria broth lacking added arabinose and since we had observed less morbidity and mortality when χ8650 with the Δ*pmi-2426* mutation was grown in Luria broth without added mannose, it has become our practice to grow the doubly mutant strain in Luria broth without added mannose or arabinose. These growth conditions yield total attenuation to inoculation with high titers of the vaccine strain and induce the highest level of protective immunity to challenge with wild-type *S. typhimurium*.

Table 7. Virulence and protection of *S. typhimurium* UK-1 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur* mutant □8754 in 8-week-old female BALB/c mice following oral inoculation[a]

Strain	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
□3761 wild-type			1.0 X 10^7	0/5
□8754 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur*	1.1 x 10^9	5/5	1.0 x 10^9	5/5
	1.1 x 10^8	5/5	1.0 x 10^9	5/5

Table 7. Virulence and protection of *S. typhimurium* UK-1 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur* mutant □8754 in 8-w ek-old female BALB/c mice following oral inoculation[a]

Strain	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
	1.1×10^7	5/5	1.0×10^9	4/5

[a] Bacteria were grown in Luria broth supplemented with 0.5% mannose and 0.2% arabinose to OD_{600} of ~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 □3761 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

Example 7. Induction of cross protective immunity to challenge with wild-type *S. enteritidis*.

Eight-week-old female BALB/c mice were orally inoculated with decreasing doses of χ8754 grown in Luria broth (without added mannose or arabinose) to an OD_{600} of approximately 0.8 and suspended in BSG. In this experiment, immunized mice were challenged 30 days later with *S. enteriditis* strain χ3700 (phage type 13a) also grown in Luria broth to an OD_{600} of approximately 0.8 and resuspended in BSG. Eighty percent of mice immunized with either the highest dose of χ8754 or with a dose of χ8754 that was 10-times less than the challenge dose of χ3700, survived challenge with χ3700 (Table 8). Mice immunized with a vaccine inoculum only 1% of the challenge inoculum were not protected (Table 8). It is therefore evident that there is a significant level of cross protective immunity induced by the group B *S. typhimurium* ΔPfur223::TT *araC* P_{BAD}*fur* Δ*pmi-2426* candidate vaccine strain to challenge with a wild-type group D *S. enteriditis* strain known to be capable of egg-transmitted disease in humans. Based on past results, it would be expected that the level of cross protective immunity would be further enhanced by a booster immunization seven or so days after the initial immunization.

Table 8. Cross protection in mice immunized with *S. typhimurium UK-1* Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur* strain □8754 and challenged with *S. enteritidis* wild-type □3700[a]

Strain	Inoculating dose	Survivors/ total	Challenge dose	Survivors/ total after challenge	MDD[b]
□3700			1.2×10^9	0/5	wild-type
□8754 Δ*pmi-2426* ΔPfur223:TT *araC*P_{BAD}*fur*	1.0×10^9	5/5	1.2×10^9	4/5	12
	1.0×10^8	5/5	1.2×10^9	4/5	14
	1.0×10^7	5/5	1.2×10^9	0/5	10.5

[a] Bacteria were grown in Luria broth to OD_{600} of ~0. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with wild-type *S. enteritidis* □3700 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

[b] MDD: Mean day of death.

Example 8. Induction of serum antibody responses against OMPs and IROMPs in diverse serotypes of *Salmonella* and in several strains of *E. coli*.

Serum antibodies were collected 30 days after oral inoculation of mice with either χ8650 with the Δ*pmi-2426* mutation or χ8634 with the ΔPfur223::TT *araC* P_{BAD} *fur* mutation by retro orbital bleeding. Serum IgG antibodies to *Salmonella* and *E. coli* OMPs and IROMPs were quantitated by ELISA. Briefly, 96-well ELISA plates were coated with OMPs or IROMPs isolated from *Salmonella* and *E. coli* strains (see below). The plates were blocked with 1 % BSA in PBS plus 0.1 % Tween 20 (blocking buffer). Serum samples were pooled from 4 mice and diluted

1:100 in blocking buffer. A volume on 100 µl of each diluted sample was added in duplicate to the 96-well plates, incubated at 37°C for 2 h and washed with PBS plus 0.05 % Tween 20. The plates were then incubated with biotin-avidin-labeled goat anti-mouse IgG (1:1000 in blocking buffer) and alkaline phosphatase-labeled Extravidin (1:4000 in blocking buffer). *p*-nitrophenylphosphate (1 mg/ml) in 0.1 M diethanolamine buffer was used as a substrate. The absorbency of the color reaction was read at 405 nm with an automated ELISA reader.

The OMPs and IROMPs as the test antigens for ELISA were isolated from bacteria of various serotypes of *Salmonella* and *E. coli* (Table 1). The bacteria were grown in Luria broth plus 200 mM $FeCl_3$ to repress synthesis of IROMPs and in Luria broth plus 200 mM α,α'-dipyridyl to sequester iron and cause IROMP synthesis to be constitutive. Bacterial cells were collected by centrifugation and the cell pellets suspended in 10 mM HEPES buffer. The cell suspension was sonicated with six 10 s pulses at 40 w. The sonicated suspension was centrifuged at 15,600 x g for 2 min at 4°C. The supernatant fluid was centrifuged again for 30 min at 4°C. The cell membrane pellets were suspended in HEPES buffer and an equal volume of 2 % Sarkosyl added. The suspension was incubated at room temperature for 30 min with gentile shaking. The suspension was then centrifuged at 15,600 x g for 30 min and the supernatant was discarded. The membrane pellets were washed with and re-suspended in HEPES buffer. The concentration of protein in each preparation was determined. Separate ELISA plates were coated with OMP and IROMP preparations (200 ng/well) from each strain used in the analysis. It should be noted that the IROMP preparations also contain OMPs.

It is evident from the data presented in Figure 11 that both bacterial vaccines induced significant titers of antibodies that react with the 0MPs present in serogroups C1, C2, C3, D and E1. In addition, significant antibody titers were induced to the OMPs of most of the *E. coli* strains with the lowest titers to the OMPs present in the totally attenuated laboratory *E. coli* K-12 strain χ289 (Figure 11).

The same serum antibodies were used to determine the antibody titers against IROMPs obtained from the same bacterial strains used in the proceeding experiment. As revealed

by the data in Figure 12, both χ8650 and χ8634 induced substantial antibody responses to the IROMPs from all strains of *Salmonella* and *E. coli* evaluated. The results of these two experiments are in accord with the evidence for cross protective immunity as revealed by challenge of immunized mice with a heterologous *S. enteriditis* group D strain (Table 8).

Example 9. Attenuation of *S. typhimurium* strains with Δ*pmi-2426* and ΔPfur::TT *araC* P_{BAD} *fur* in day-of-hatch white leghorn chicks.

Results presented in Table 9 indicate that *S. typhimurium* strain χ8754 is completely attenuated when used to inoculate day-of-hatch chicks at doses in excess of 1 x 10^9 CFU. For these experiments, the day-of-hatch chicks were infected before being provided with either food or water. These white leghorn chicks are hatched in our animal facility from fertile eggs obtained from SPAFAS. Bacteria for infection are grown in Luria broth and concentrated in BSG in the same manner as used for experiments to infect mice as described above. In this experiment, the LD_{50} for χ8754 was in excess of 4 X 10^9 (Table 9). The same result was observed with χ8754 grown in Luria broth without added mannose and arabinose (data not shown). However, some chicks survived infection with 1 X 10^7 CFU of the wild-type χ3761, a dose that is far in excess of the LD_{50}. This result is sometimes observed due to a very rapid stimulation of a protective innate immune response by the high inoculating dose of virulent bacteria. This type of response is seen more often in birds that are naturally more refractory to infection by *Salmonella* than in inbred mice. Results are also more variable since the chickens are out bred and we do not get fertile eggs from the same flock of breeders for each shipment from SPAFAS.

Table 9. Virulence of S. typhimurium UK-1 Δ*pmi-2426* ΔPfur223::TT*araC* P_{BAD} *fur* mutant χ8754 in day-of-hatch chicks following oral inoculation

Strains/Genotype	Inoculation Dose (cfu)	Survivors/total	LD50
χ8754/ΔPfur::araC	4.3 x 10^9	4/4	> 4 X 10^9

Table 9. Virulence of S. typhimurium UK-1 Δ*pmi-2426* ΔPfur223::TT*araC* P_{BAD}*fur* mutant χ8754 in day-of-hatch chicks following oral inoculation

Strains/Genotype	Inoculation Dose (cfu)	Survivors/total	LD50
PBADfur11			
	2.3×10^9	4/4	
	1.3×10^9	4/4	
χ3761/wild-type	1.2×10^7	2/4	

Example 10. Ability of candidate vaccine strains to colonize and persist in lymphoid tissues of vaccinated chicks.

Day-of-hatch chicks were orally inoculated with the candidate vaccine strain χ8754 grown in L broth to an OD_{600} of 0.8 and suspended in BSG. Groups of chicks were euthanized on various days after initial infection to quantitate the titers of χ8754 in the bursa of Fabricius, the spleen and in cecal contents. Results of these studies are presented in Figure 13. The increases in titers at 28 days after inoculation were unusual and unexpected. However, in the evaluation of the ability of χ8754 to colonize mice, the titers dropped significantly after 28 days (Figure 9).

Example 11. Introduction of Δ*fliC825* and Δ*fljB217* mutations into the candidate vaccine strain χ8754.

The various *Salmonella* serotypes generally have genetic information to express two antigenically different flagellar antigens (a minority express only one) and employ a genetic switching mechanism for phase variation to express one or the other flagellar antigenic type. Since the flagellar antigens are very immunogenetic and since there is great diversity of antigenic flagellar types in enteric bacteria infecting the intestinal tract that do not exhibit a significant degree of antigenic similarity, we have deleted the genes for the *S. typhimurium fliC* and *fljB* flagellar antigens. This decision was based on the fact that antibodies to the FliC and FljB

flagellar antigens would not be of significance in inducing cross protective immunity and that induction of immune responses to these antigens would compete with the induction of antibody responses to the common LPS core antigen or to the highly cross reactive OMP and IROMP surface protein antigens that are important for induction of cross protective immunity. The construction of the suicide vector pYA3547 for introduction of the Δ*fliC825* mutation into the chromosome is shown in Figure 14. The construction of the suicide vector pYA3548 for introduction of the Δ*fljB217* mutation into the chromosome is shown in Figure 15. The molecular genetic attributes of the Δ*fliC825* and Δ*fljB217* mutations upon introduction into the chromosome are depicted in Figure 16. Both of these suicide vectors are transferred to MGN-617 (Table 1) and the constructed strains used for conjugational transfer of the suicide vectors to χ8754 possessing the Δ*pmi-2426* and ΔPfur::TT *araC* P_{BAD} *fur* mutations. In the first step, transfer by MGN-617 of pYA3547 to χ8754 followed by selection for chloramphenicol resistance yields recombinants with the suicide vector integrated into the chromosome. These chloramphenicol-resistant recombinants are then grown in L broth in the absence of chloramphenicol and subjected to selection for sucrose-resistant isolates by plating on L agar containing 5 % sucrose. This selection results in loss of the integrated suicide vector by a second reciprocal crossing over event to often result in allele replacement with inheritance of the Δ*fliC825* mutation in place of the wild-type allele. The Δ*flgB217* allele is introduced in the same way starting with the transfer by MGN-617 of the suicide vector pYA3548 and its subsequent integration (by selecting for tetracycline resistance) into and then excision (by selecting for sucrose resistance) from the chromosome for allele replacement. Following construction, strains are evaluated to demonstrate the absence of motility and the absence of flagellar antigens by a negative slide agglutination test with the Difco antisera against *Salmonella* flagellar antigens used previously (see Example 4). The presence of all four mutational alterations can be validated by PCR analyses and conduct of tests for the phenotype associated with each mutation as described in previous examples.

Example 12. Evaluation of induction of cross protective immunity in chickens.

Experiments to evaluate induction of cross protective immunity against diverse *Salmonella* serotypes is by a slight modification of the methods worked out and described by Hassan and Curtiss (1994, Infect, Immun. 62:5519-5527). Day-of-hatch chicks are immunized orally with 10^8 CFU of the vaccine described in Example 11 above with a booster immunization of the same dose administered 10 days later. These chicks and groups of unimmunized chicks as controls are challenged with *Salmonella* of numerous serotypes as listed in Table 1. Vaccine and challenge strains are grown in Luria broth and resuspended in BSG before oral inoculation. Groups of five challenged birds are euthanized 7 and 14 days after challenge and the titers of the challenge strain in the bursa of Fabricius, spleen, liver, ovaries and in the contents of the small intestine (ileum) and cecum determined. To evaluate induction of cross protective immunity against APEC infection, the APEC challenge strains can be administered by injection into the caudal air sac or by intratracheal inoculation.

Example 13. Construction of mutant derivatives of host-specific *Salmonella* serotypes for use as vaccines to induce cross protective immunity to gram-negative enteric pathogens in swine, cattle and humans.

S. choleraesuis is a host-adapted *Salmonella* that predominantly infects swine. *S. dublin* is a host-adapted *Salmonella* that predominately infects cattle. *S. paratyphi* A and *S. typhi* are host-adapted *Salmonella* that predominantly infect humans. The suicide vectors and methods for introducing the Δ*pmi-2426* and ΔPfur::TT *araC* P_{BAD} *fur* mutations are the same as described in the Examples given above. Each of these *Salmonella* serotypes possesses unique genes for the predominant flagellar antigens. Therefore, specific suicide vectors based on DNA sequence information for the flagellar genes in each of these serotypes is used to generate deletions for both flagellar antigen genes in each of the serotypes. The *S. choleraesuis* χ3246, *S. dublin* χ4860,*S. paratyphi* A χ8387 and *S. typhi* χ3744 and χ8438 strains that are altered by these genetic manipulations are listed in Table 1. The presence of each of the mutations can be ascertained by PCR analyses and testing for the specific phenotype associated with each

[redacted]utation. Difco antisera is used to verify the presence of the appropriate group A, C1 or D O-antigens. The *S. choleraesuis* and *S. dublin* vaccines can initially be evaluated for induction of cross protective immunity in mice using challenge of immunized mice with a diversity of *Salmonella* strains of different serotypes (Table 1) as well as with other gram-negative enteropathogens. Subsequent evaluations would use pigs and calves to substantiate induction of cross protective immunity by the candidate *S. choleraesuis* and *S. dublin* vaccines, respectively. The *S. paratyphi* A and *S. typhi* candidate vaccines will be evaluated in human volunteers since there is no suitable animal model.

CLAIMS

What is claimed is:

1. A live attenuated derivative of a pathogenic Enterobacteriaceae species, consisting essentially of

(a) a means for regulatable expression of a gene that encodes a regulatory protein, wherein expression of said regulatory protein in vivo causes synthesis of antigenic proteins that are conserved among Enterobacteriaceae; and

(b) a means for regulatable synthesis of a second antigen, wherein said second antigen ceases to be synthesized in vivo, exposing a carbohydrate antigen that is conserved among Enterobacteriaceae;

wherein said attenuated derivative has enhanced ability to induce cross protective immunity against Enterobacteriaceae.

2. The live attenuated derivative of claim 1, wherein said means of regulatable expression comprises substituting the promoter of said gene that encodes a regulatory protein with a regulatable promoter.

3. The live attenuated derivative of claim 2 wherein said regulatable promoter is the *araC*P_{BAD} repressor-activator-promoter system.

4. The live attenuated derivative of claim 3 wherein said carbohydrate antigen is an LPS O-antigen.

5. The live attenuated derivative of claim 4 wherein said means for regulatable synthesis comprises a mutation in a gene that encodes a product necessary for synthesis of LPS O-antigen.

6. The live attenuated derivative of claim 5, wherein said means for regulatable synthesis comprises a mutation in the *pmi* gene.

7. A method for inducing an immune response sufficient for protection against infection by Enterobacteriaceae species, said method comprising administering to an individual the live attenuated derivative of any one of claims 1-6.

8. A live attenuated derivative of a pathogenic Enterobacteriacea species, consisting essentially of

(a) a means for regulatable expression of a *fur* gene; and

(b) a mutation that renders a *pmi* gene inoperable,

wherein said attenuated derivative has enhanced ability to induce cross protective immunity against Enterobacteriaceae.

9. The live attenuated derivative of claim 8 wherein said means of (a) comprises substituting the *fur* promoter with a regulatable promoter.

10. The live attenuated derivative of claim 8, wherein said means of (a) comprises replacing the *fur* promoter with the *araC*P_{BAD} activator-repressor-promoter system.

11. The live attenuated derivative of claim 8 wherein said means of (a) comprises the ΔPfur223::*araC*P_{BAD} genetic construction.

12. The live attenuated derivative of claim 8 wherein said mutation of (b) is a deletion mutation.

13. A method of inducing a cross-protective immune response against Enterobacteriaceae species, said method comprising administering to an individual the live attenuated derivative of any of claims 8-12.

14. A live attenuated derivative of a pathogenic Enterobacteriaceae consisting essentially of

(a) a means for regulatable expression of a first surface antigen, wherein said first surface antigen is conserved among Enterobacteriaceae; and

(b) a means for regulatable expression of a second surface antigen, wherein said second surface antigen is not conserved among Enterobacteriaceae,

wherein up regulation of said first surface antigen and down regulation of said second surface antigen results in enhanced ability of said attenuated derivative to produce immunity against Enterobacteriaceae.

15. A vaccine comprising a live attenuated strain of *Salmonella*, wherein said live attenuated strain consists essentially of

(a) a mutation in a *pmi* gene that renders said *pmi* gene non functional; and;

(b) a genetic construction that allows for regulatable expression of a *fur* gene,

wherein said vaccine has enhanced ability to stimulate cross protective immunity against Enterobacteriaceae.

16. A method for inducing an immune response to Enterobacteriaceae comprising administering to an individual a live attenuated derivative of a pathogenic Enterobacteriaceae that is capable of colonizing the intestinal tract and reaching and persisting in the Gut Associated Lymphoid Tissue, and wherein expression of at least one conserved surface antigen is up regulated and at least one non-conserved surface antigen is down regulated in said attenuated derivative when said attenuated derivative is in the lymphoid tissue of the individual, wherein said live attenuated derivative has enhanced ability to stimulate cross protective immunity against infection by Enterobacteriaceae.

17. A vaccine comprising a live attenuated strain of *Salmonella*, wherein said live attenuated strain consists essentially of

(a) a mutation that renders a *pmi* gene non functional; and

(b) a regulatable promotor operably linked to a *fur* gene wherein said *fur* gene is expressed when said attenuated strain is in the intestinal tract of an individual and said *fur* gene is not expressed when said attenuated strain is in the lymphoid tissue of an individual.

18. The vaccine of claim 17 wherein said regulatable promoter comprises the *araC*P_{BAD} activator-repressor-promoter system.

19. A live attenuated derivative of an Enteropathogenic bacteria consisting essentially of

(a) a means for regulatable synthesis of LPS O-antigen side chains, wherein said O-antigen side chains are synthesized when said attenuated derivative is in the intestinal tract of an individual and are not synthesized when said attenuated derivative is in the lymphoid tissue of an individual; and

(b) a means for regulatable expression of a *fur* gene, wherein said *fur* gene is expressed when said attenuated derivative is in the intestinal tract of an individual and wherein said *fur* gene is not expressed when said attenuated derivative is in the lymphoid tissue of an individual

wherein said attenuated derivative has increased ability to induce cross protective immunity against infection from Enterobacteriaceae.

20. The live attenuated derivative of claim 19 wherein said means for regulatable synthesis comprises a mutation in a gene that encodes a product necessary for synthesis of LPS O-antigens.

21. The live attenuated derivative of claim 20 wherein said gene that encodes a product necessary for synthesis of LPS O-antigens is a *pmi* gene.

22. A live attenuated derivative of a *Salmonella typhimurium* comprising

(a) a ΔPfur223::TT*araC*P_{BAD}*fur* deletion-insertion mutation; and

(b) a Δ*pmi* mutation

23. A recombinant bacterial strain consisting essentially of a means of regulatable expression of a virulence gene, wherein said regulatable expression of a virulence gene renders said bacterial strain attenuated while maintaining immunogenicity.

24. The recombinant bacterial strain of claim 23, wherein said means of regulatable expression comprises substituting the promoter for said virulence gene with the *araC*P_{BAD} repressor-activator-promoter system.

25. The recombinant bacterial strain of claim 24, wherein said virulence gene is a *fur* gene.

26. The recombinant bacterial strain of claim 25, wherein said bacterial strain is a strain of *Salmonella*.

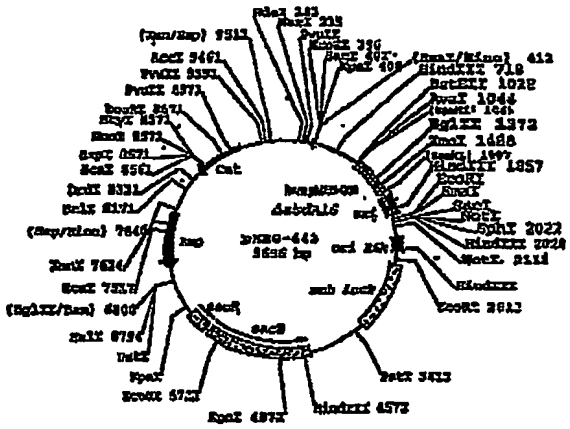
27. The recombinant bacterial strain of claim 26, further comprising a Δ*pmi* mutation.

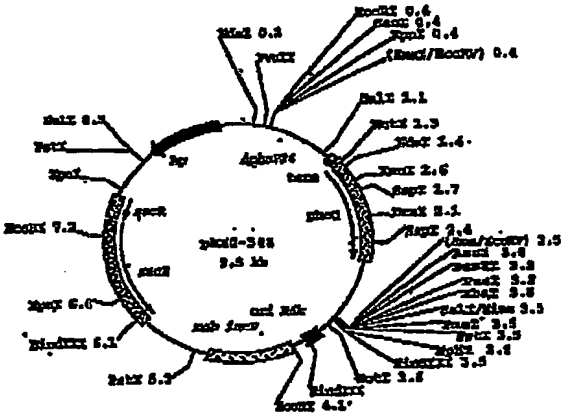
28. A live attenuated derivative of a pathogenic *Enterobacteriaceae* species consisting essentially of a ΔPfur223::*araC*P_{BAD}*fur* genetic construction.

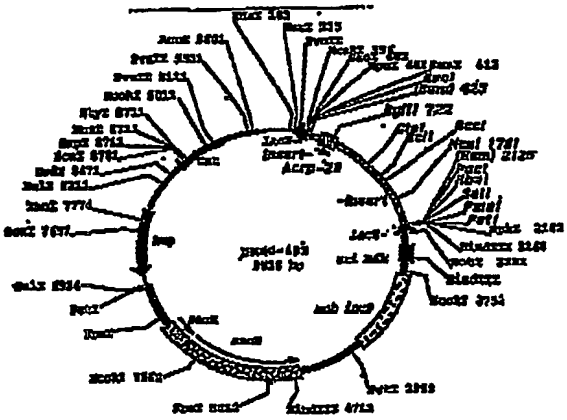
29. The live attenuated derivative of claim 28, wherein said species is *Salmonella*.

Title: Regulated Attenuation of Live Vaccines to Enhance Cross-protective Immunogenicity
Inventor(s): Kang et al.
Express No. El474185957US
Docket # 56029/31335

FIGURE 1.







Title: Regulated Attenuation of Live Vaccines to Enhance Cross-protective Immunogenicity
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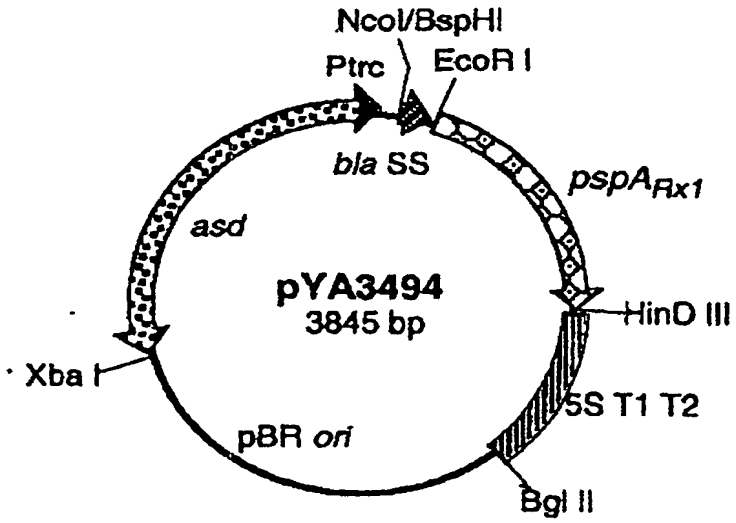

FIGURE 2

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Title: Regulated Attenuation of Live Vaccines to Enhance Cross-protective Immunogenicity

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FIGURE 3. PspA specific IgG1 and IgG2a responses induced by live *S. typhimurium* vaccines with different types of attenuation

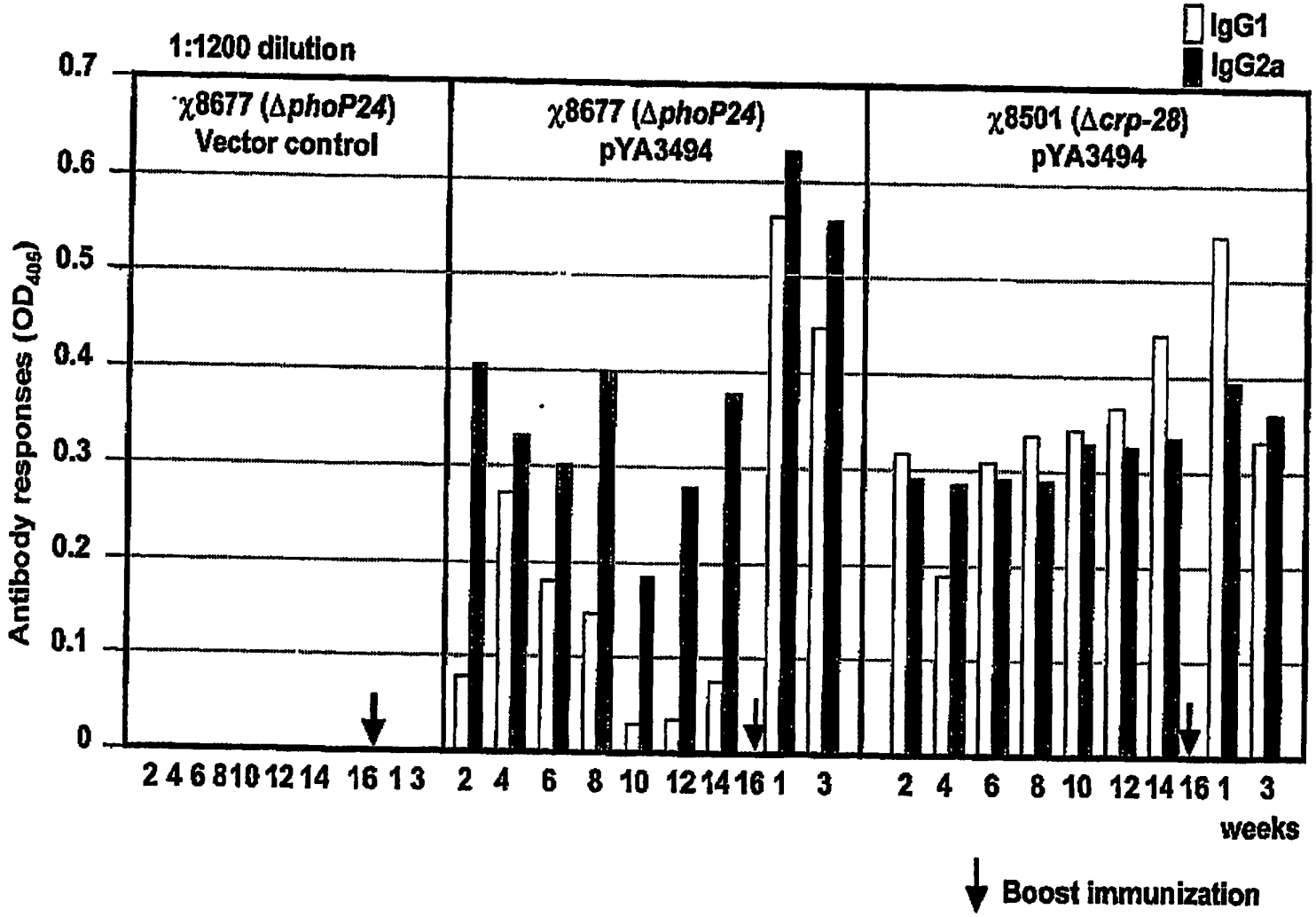

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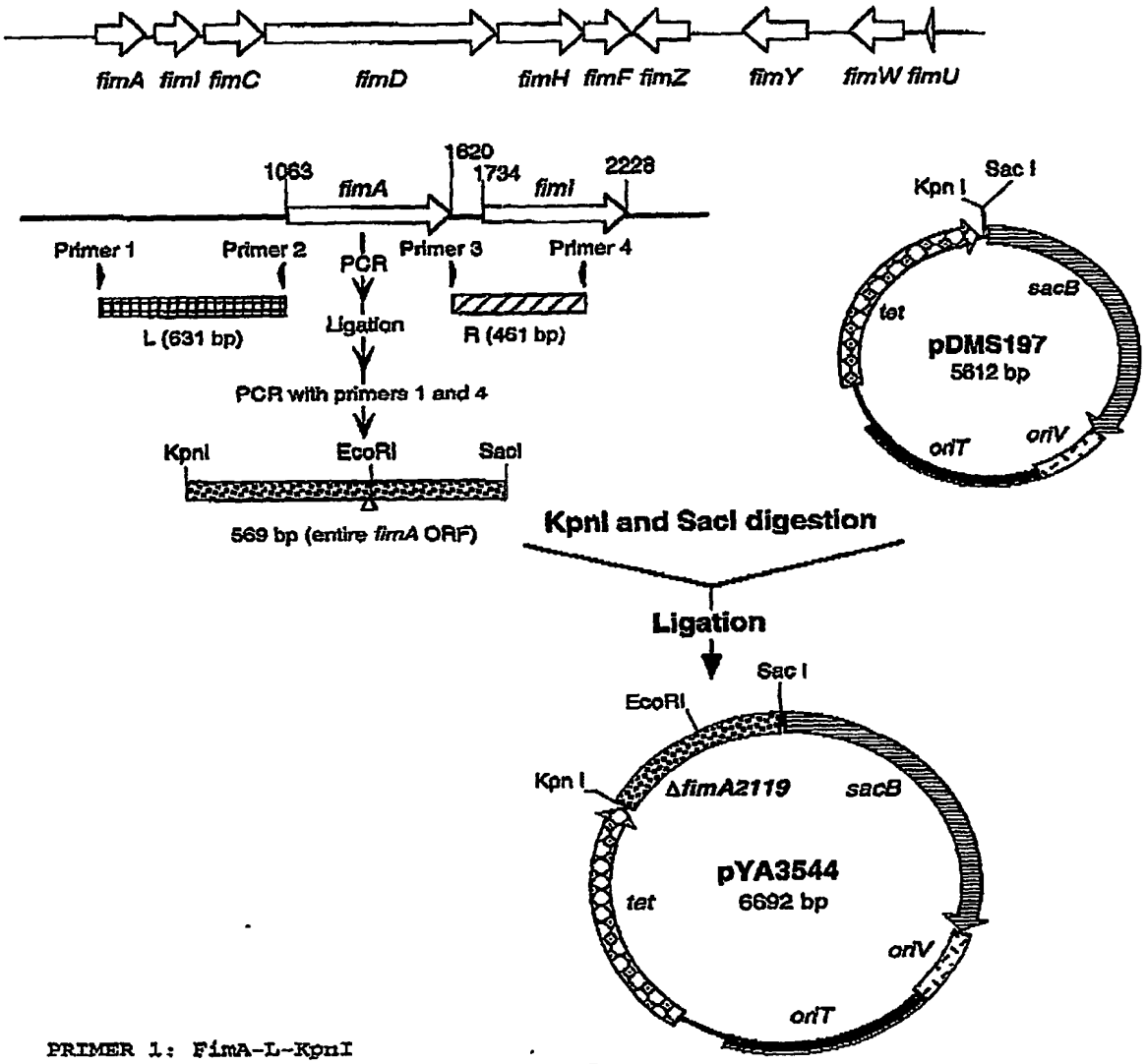
FIGURE 4. Construction of sui ide vector f r Δ*fimA2119*



PRIMER 1: FimA-L-KpnI
5' GGG GTA CCC GAA GAC CTG CTG CGA C 3'

PRIMER 2: FimA-L-EcoRI
5' CCG AAT TCA ATT ACA CAC ACC CGG T 3'

PRIMER 3: FimA-R-EcoRI
5' CCG AAT TCA TCC CGT CAG GGA ACG G 3'

PRIMER 4: FimA-R-SacI
5' GCG AGC TCA TTT GCC GCT GCT GGT C 3'

Title: Regulated Attenuation of Live Vaccines to Enhan Cross-protective Immunogenicity
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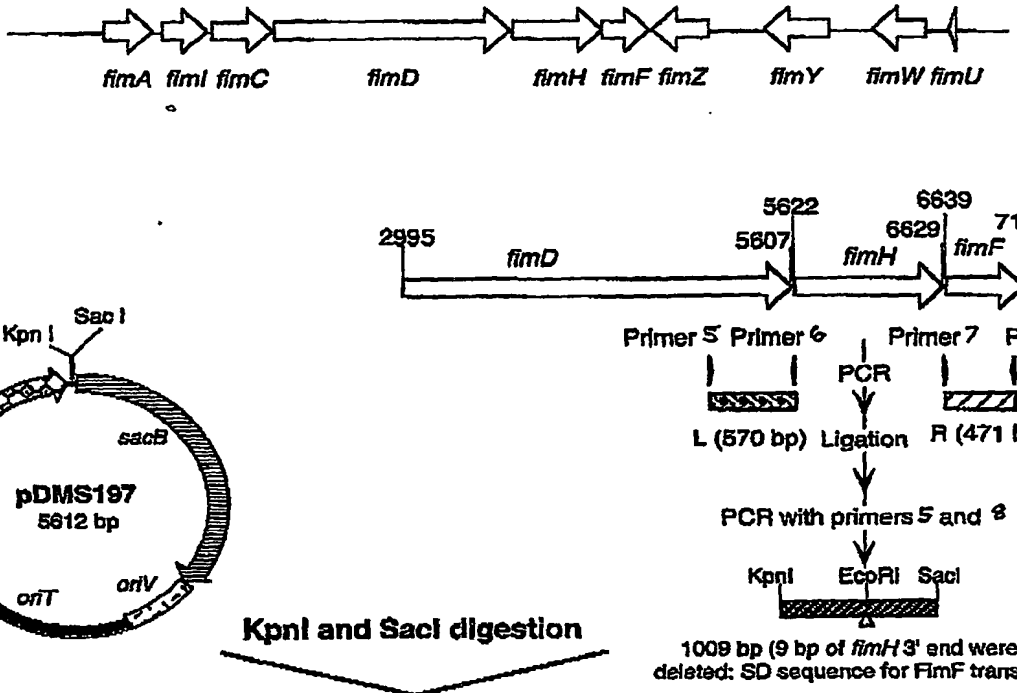
FIGURE 5. Constructi n f suicid vector for Δ*fimH1019*



PRIMER 5: FimH-L-KpnI
5' GGG GTA CCT GGC AAC TTT CCG GCG G 3'

PRIMER 6: FimH-L-EcoRI
5' CCG AAT TCC TTT TAT GAC GCC GGA C 3'

PRIMER 7: FimH-R-EcoRI
5' CCG AAT TCT TAT GAT TAA GGA GGC A 3'

PRIMER 8: FimH-R-SacI
5' GCG AGC TCG CGA TAT AGT TCG CAT A 3'

Title: Regulated Attenuation of Live Vaccines to Enha... Cross-protective Immunogenicity
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FIGURE 6. Construction of suicid vector for Δ*fimW113*

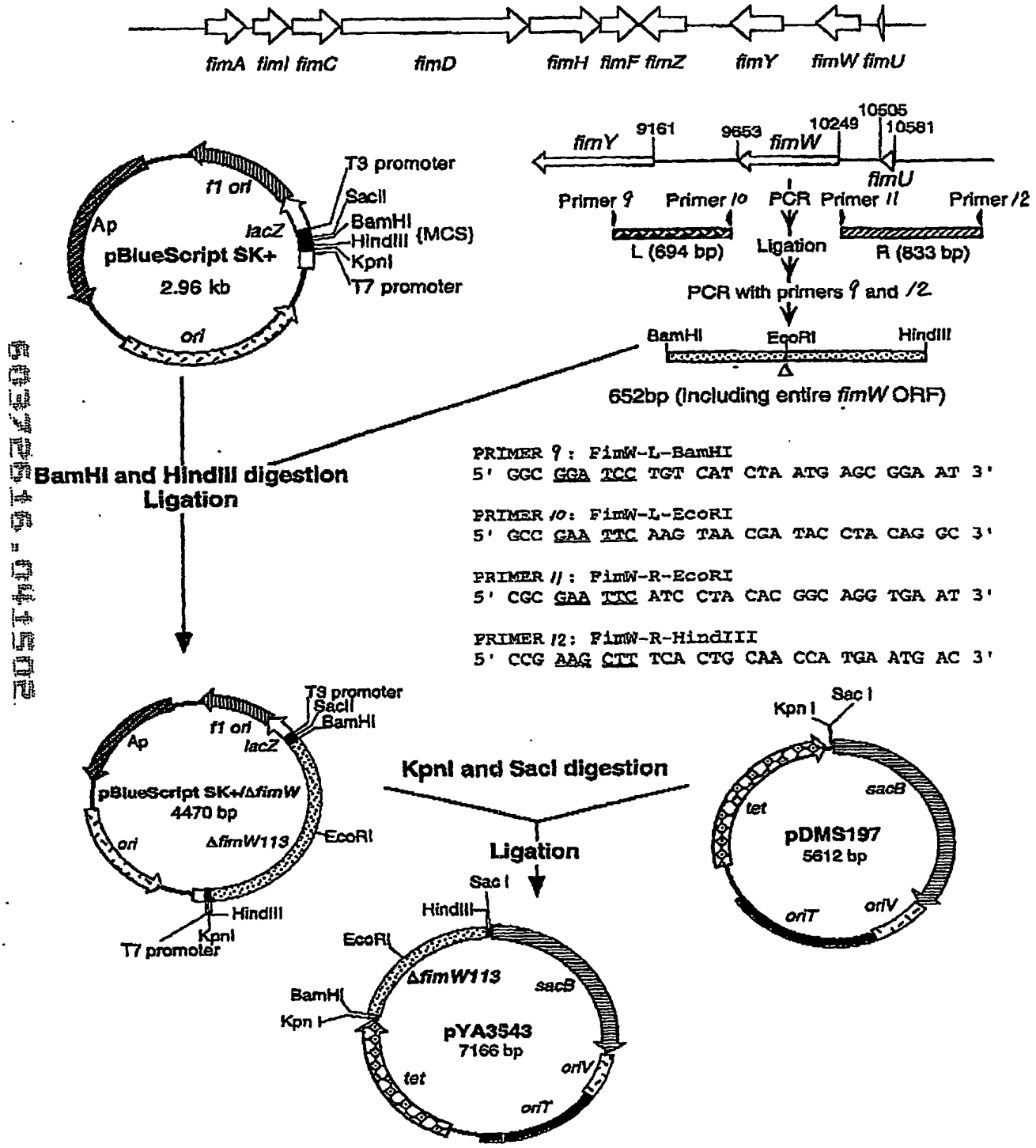
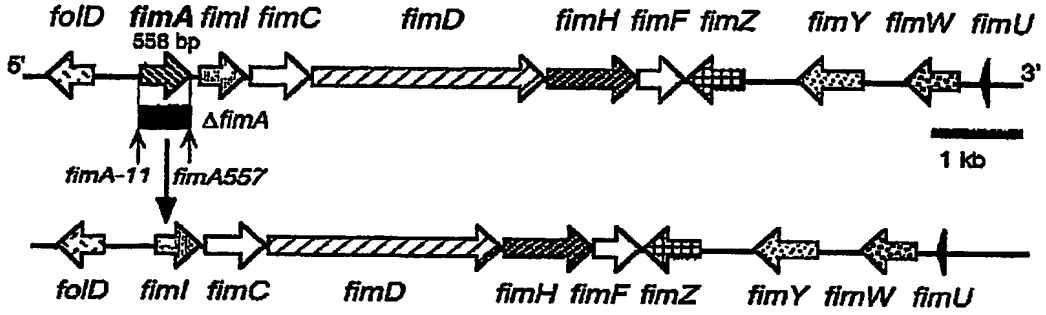


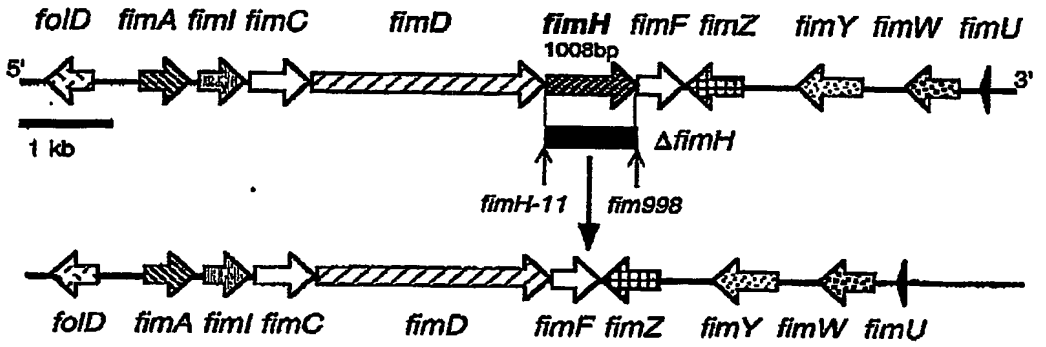
FIGURE 7

A. Δ*fimA2119*



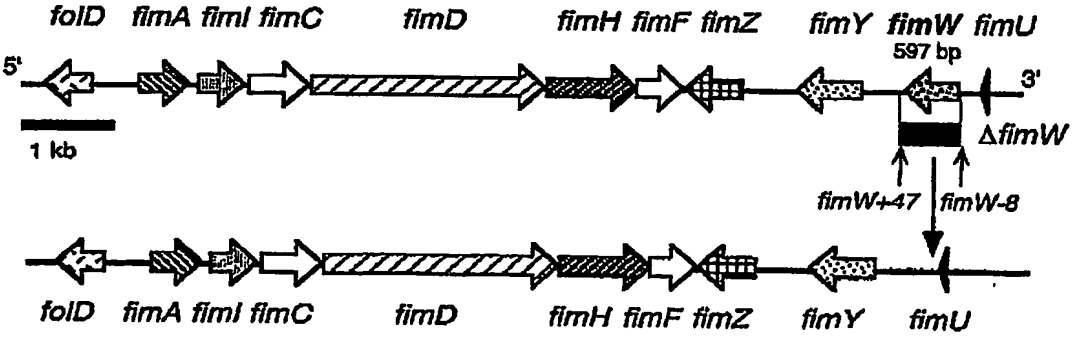
Δ*fimA2119*
569 bp deleted including 558 bp *fimA* ORF

B. Δ*fimH1019*



Δ*fimH*
1009 bp (9 bp of *fimH* 3' end were not deleted:
SD sequence for FimF translation)

C. Δ*fimW113*



Δ*fimW*: 652 bp deleted including
entire597 bp *fimW* ORF

Title: Regulated Attenuation of Live Vaccines to Enhance Cross-protective Immunogenicity
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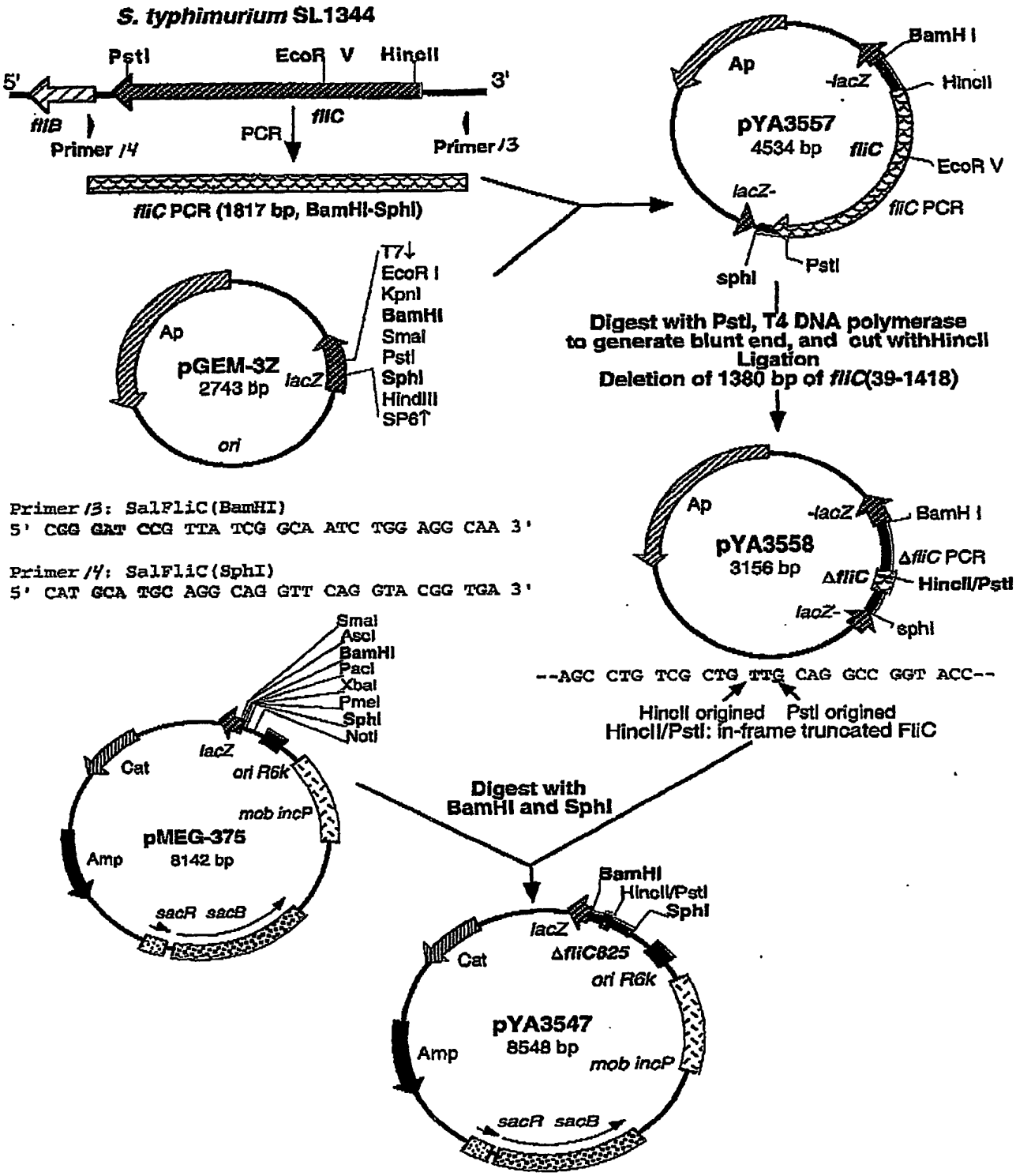
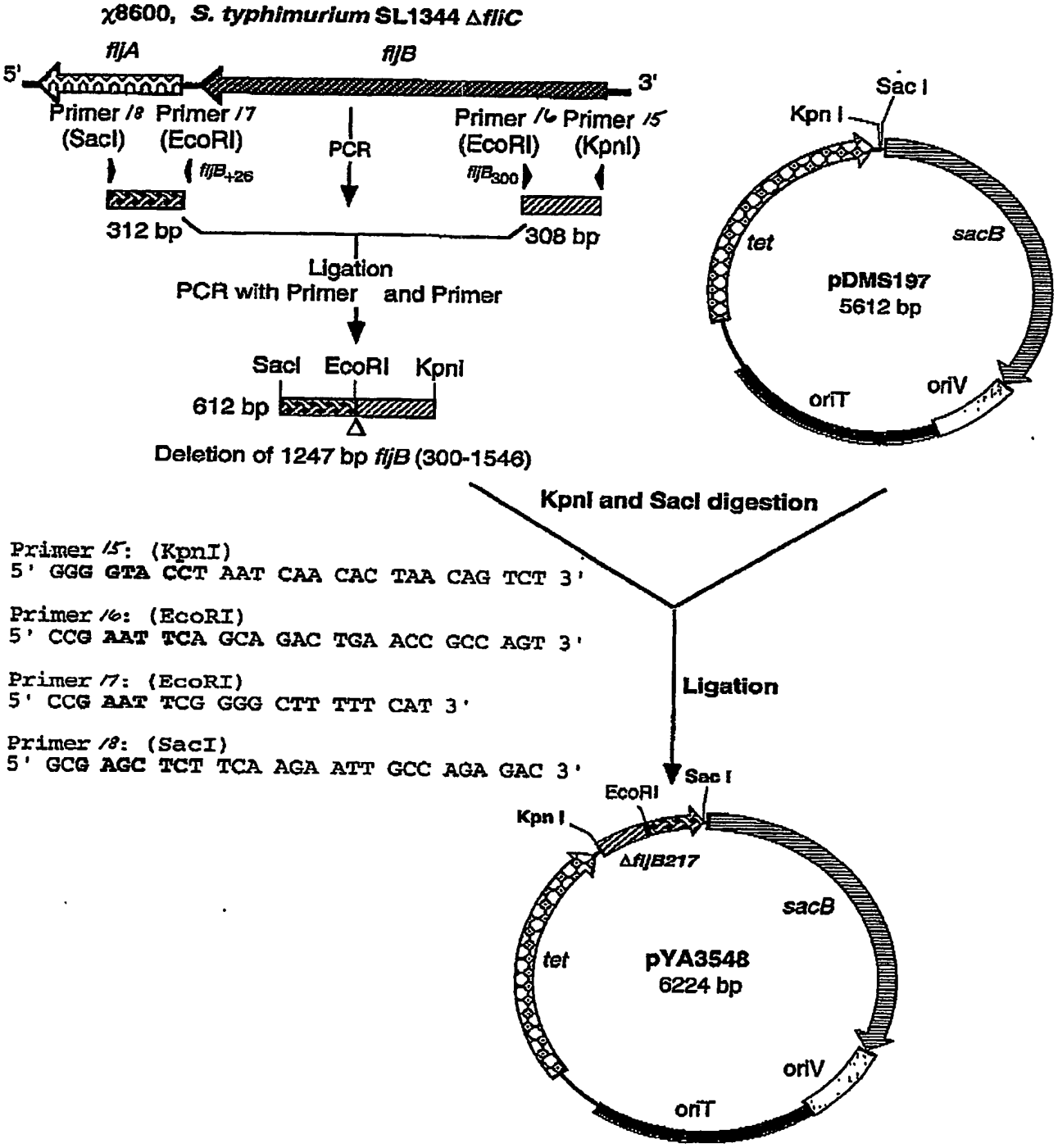
Construction of suicid vector for Δ*fliC825*

FIGURE 8



Construction of uicid vector for Δ*fljB217*

FIGURE 9



Title:	Regulated Attenuation of Live Vaccines to Enhance Cross-protective Immunogenicity
Inven	Kang et al.
	Express El474185957US
Docket #	56029/31335

FIGURE 10

Salmonella typhimurium SL1344 chromosomal deletions:

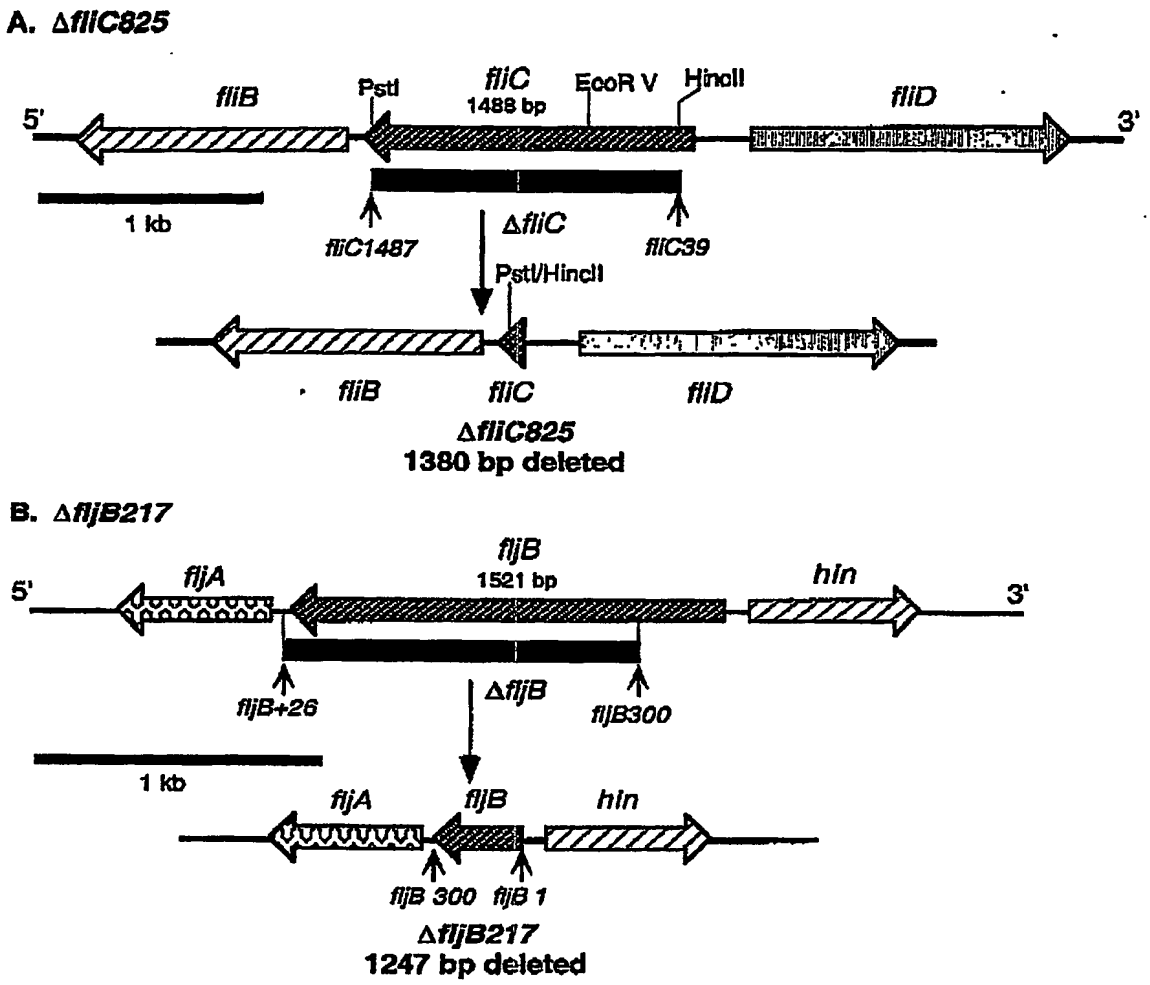
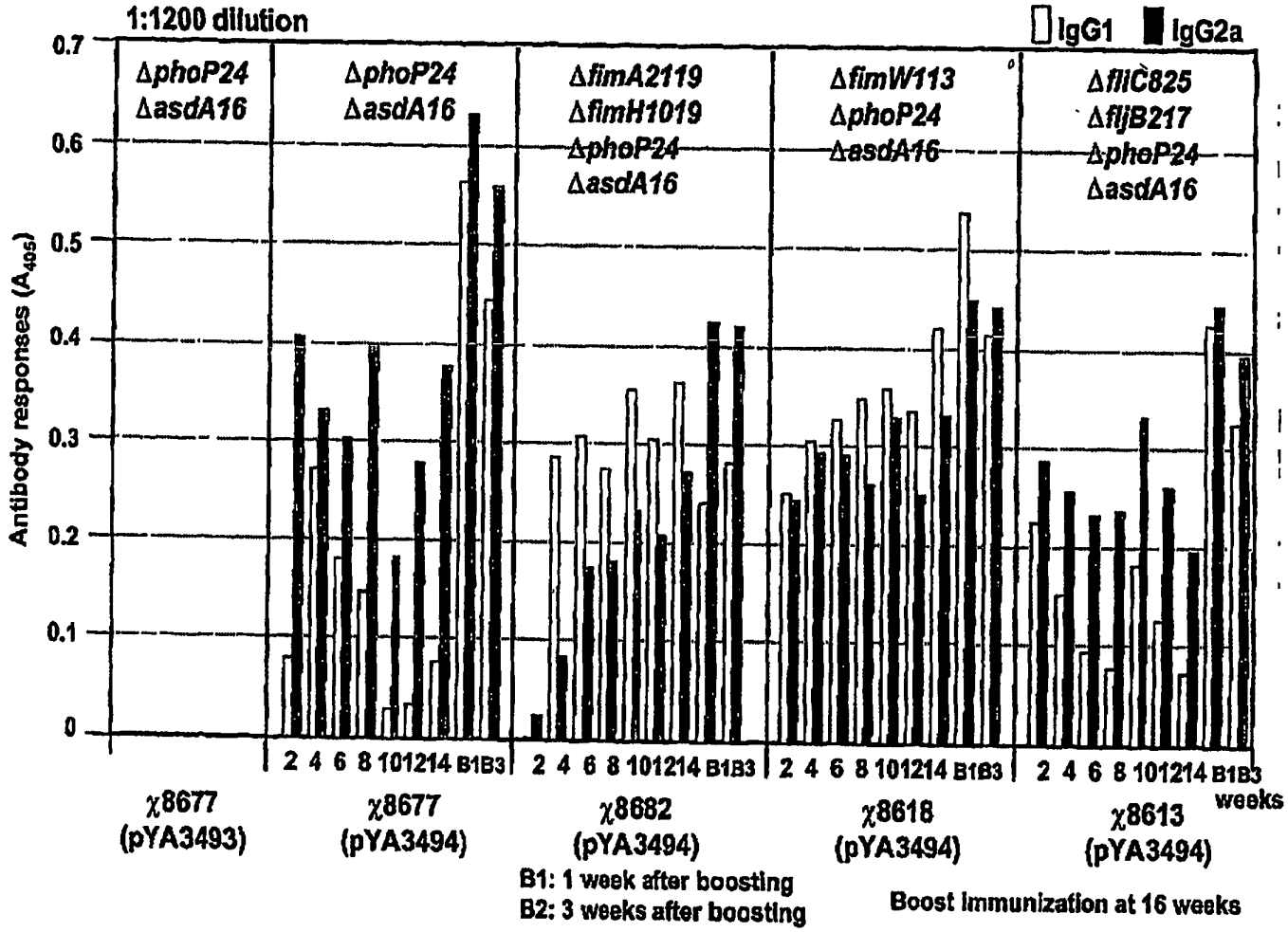


FIGURE 11. PspA specific IgG1 and IgG2a responses induced by live *S. typhimurium* vaccines with various adhesion mutants



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Lys Ser Glu Glu Ala Ala Lys Glu Val Glu Val Glu Lys Asn Lys Ile
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Ala Ile Thr Asn Lys Lys Ala Glu Leu Ala Thr Thr Gln Gln Asn Ile
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Val Leu Ala Thr Leu Asp Pro Glu Gly Lys Thr Gln Asp Glu Leu Asp
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Tyr Gln Gln Ala Thr Asp Lys Ala Ala Lys Asp Ala Ala Asp Lys Met
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Ile Asp Glu Ala Lys Lys Arg Glu Glu Glu Ala Lys Thr Lys Phe Asn
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Thr Val Arg Ala Met Val Val Pro Glu Pro Glu Gln Leu Ala Glu Thr
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Lys Ile Ala Glu Leu Glu Asn Gln Val His Arg Leu Glu Gln Glu Leu
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Lys Glu Ile Asp Glu Ser Glu Ser Glu Asp Tyr Ala Lys Glu Gly Phe
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Arg Ala Pro Leu Gln Ser Lys Leu Asp Ala Lys Lys Ala Lys Leu Ser
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Ile Arg Thr Thr Ile Val Val Pro Glu Pro Ser Glu Leu Ala Glu Thr
35 40 45

Lys Lys Lys Ala Glu Glu Ala Thr Lys Glu Ala Glu Val Ala Lys Lys
50 55 60

Lys Ser Glu Glu Ala Ala Lys Glu Val Glu Val Glu Lys Asn Lys Ile
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Leu Glu Gln Asp Ala Glu Asn Glu Lys Lys Ile Asp Val Leu Gln Asn
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Lys Lys Ala Lys Leu Ser Lys Leu Glu Glu Leu Ser Asp Lys Ile Asp
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